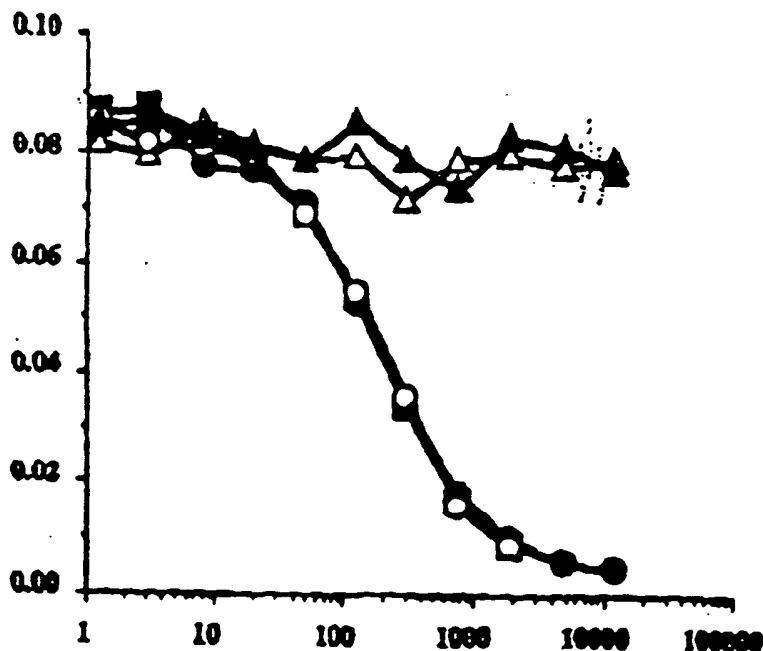




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(54) Title: METHODS AND MATERIALS FOR MODULATION OF THE IMMUNOSUPPRESSIVE ACTIVITY AND TOXICITY OF MONOCLONAL ANTIBODIES



(57) Abstract

The binding specificity of the murine OKT3 has been transferred into a human antibody framework in order to reduce its immunogenicity. "Humanized" anti-CD3 mAbs, such as gOKT3-5 and gOKT3-7, have been shown to retain, *in vitro*, all the properties of native OKT3, including T cell activation which has been correlated, *in vivo*, with the severe side-effects observed in transplant recipients after the first administration of the mAb. Disclosed are modified versions of humanized anti-CD3 mAbs that do not have the property of T cell activation. Further disclosed are methods of using such mAbs.

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-1-

DESCRIPTION

METHODS AND MATERIALS FOR MODULATION OF THE
IMMUNOSUPPRESSIVE ACTIVITY AND
TOXICITY OF MONOCLONAL ANTIBODIES

Field of the Invention

10 This invention relates generally to methods and materials for modulation of the immunological activity and toxicity of immunosuppressive agents derived from murine OKT3 used in organ transplantation and in the treatment of auto-immune diseases.

Background of the Invention

15 OKT3 is a murine monoclonal antibody (mAb) which recognizes an epitope on the ϵ -subunit within the human CD3 complex (Salmeron, 1991; Transy, 1989; *see also*, U.S. Patent No. 4,658,019, herein incorporated by reference). Studies have demonstrated that OKT3
20 possesses potent T cell activating and suppressive properties depending on the assay used (Landgren, 1982; Van Severter, 1987; Weiss, 1986). Binding of OKT3 to the TcR results in coating of the TcR and or modulation, thus mediating TcR blockade, and inhibiting alloantigen recognition and cell-mediated cytotoxicity. Fc receptor-mediated cross-
25 linking of TcR-bound anti-CD3 mAb results in T cell activation marker expression, and proliferation (Weiss, 1986). Similarly, *in vivo* administration of OKT3 results in both T cell activation and suppression of immune responses (Ellenhorn, 1992; Chatenoud, 1990). Repeated daily administration of OKT3 results in profound immunosuppression, and
30 provides effective treatment of rejection following renal transplantation (Thistlethwaite, 1984).

-2-

The production of an immune response to rodent mAbs is a major obstacle to their therapeutic use. Several groups have reported attempts to circumvent this problem by reconstructing the rodent antibody genes by replacing immunogenic murine constant region sequences by the equivalent human antibody sequences (reviewed in Adair, 1992). However, in cases such as these there is still the potential to mount an immune response against the variable region. In a further extension of the procedure, the variable region framework regions have been replaced with equivalent sequences from human variable region genes. From an examination of available X-ray structures of antigen-antibody complexes (reviewed in Poljak, 1991) it is probable that only a small number of antibody residues make direct contact with antigen. Other amino acids may contribute to antigen binding by positioning the contact residues in favorable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains. Antibody domains have been the subject of detailed examination. (See for example, Looney, 1986, and references therein.)

The use of OKT3 is limited by problems of "first dose" side effects, ranging from mild flu-like symptoms to severe toxicity, which are believed to be caused by lymphokine production stimulated by OKT3. Although successful reuse of OKT3 has been reported (Woodle, 1991) it is complicated by a human anti-mouse antibody (HAMA) response (OMTSG, 1985), a proportion of the response being directed to the variable region of the antibody (Jaffers, 1984). While low titre HAMA may present no significant problem, some patients do develop high titre anti-isotype and/or anti-idiotypic responses. These can result in specific inactivation and/or the rapid clearance of the drug.

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Reported side effects of OKT3 therapy include flu-like symptoms, respiratory distress, neurological symptoms, and acute tubular necrosis

-3-

that may follow the first and sometimes the second injection of the mAb (Abramowicz, 1989; Chatenoud, 1989; Toussaint, 1989; Thistlethwaite, 1988; Goldman, 1990). It has been shown that the activating properties of OKT3 result from TCR cross-linking mediated by the mAb bound to T cells (via its F(ab')₂ portion) and to Fc γ R-bearing cells via its Fc portion) (Palacios, 1985; Ceuppens, 1985; Kan, 1986). Thus, before achieving immunosuppression, OKT3 triggers activation of mAb-bound T cells and Fc γ R-bearing cells, resulting in a massive systemic release of cytokines responsible for the acute toxicity of the mAb (Abramowicz, 1989; Chatenoud, 1989). Data obtained using experimental models in chimpanzees and mice have suggested that preventing or neutralizing the cellular activation induced by anti-CD3 mAbs reduces the toxicity of these agents (Parleviet, 1990; Rao, 1991; Alegre, *Eur. J. Immunol.*, 1990; Alegre, *Transplant Proc.*, 1990; Alegre, *Transplantation*, 1991; Alegre, *J. Immunol.*, 1991; Ferran, *Transplantation*, 1990). In addition, previous results reported in mice using F(ab')₂ fragments of 145-2C11, a hamster anti-mouse CD3 that shares many properties with OKTS3, have suggested that, in the absence of Fc γ R binding and cellular activation, anti-CD3 mAbs retain at least some immunosuppressive properties *in vivo* (Hirsch, *Transplant Proc.*, 1991; Hirsch, *J. Immunol.*, 1991).

A great need exists for nonactivating forms of anti-human CD3 mAbs for use as immunosuppressive agents.

Initial attempts to find nonactivating anti-human CD3 mAbs for use in man, involved treatment of kidney allograft recipients undergoing rejection with T10B9.1A-31, a nonmitogenic anti-TCR $\alpha\beta$ mAb. This resulted in a reduced incidence of fever as well as neurological and respiratory side effects (Lucas, 1993; Waid, 1992; Waid, 1991). However, some T cell activation or related side effects remained perhaps due to the specificity of this antibody. In addition, being an IgM mAb, the

-4-

clearance of T10B9.1A-31 is more rapid than that of OKT3 (an IgG2m mAb), thus requiring frequent injections of high doses of mAb.

Early data on the utility of chimeric antibodies (Morrison, 1984) in
5 which the coding sequences for the variable region of the mAb is retained
the coding sequences for the constant regions are derived from human
antibody suggested that the HAMA response may indeed be reduced,
however a HAMA response to the murine variable region could still
emerge (reviewed by Adair, 1992) and more recently the humanization
10 process has been taken further by substituting into a human antibody
those amino acids in the variable regions believed to be involved in
antigen binding to give a fully humanized antibody (Reichman, 1988).

A major concern is that a humanized antibody will still be
15 immunogenic because of the presence of the non-CDR residues which
need to be transferred in order to regenerate suitable antigen binding
activity, in addition to any antiparatope antibodies that may be generated.
Humanized antibodies, such as CAMPATH-1H and Hu2PLAP, have been
administered to patients (LoBuglio, 1989). Both of these antibodies used
20 the rodent amino acid sequences in CDRs as defined by Kabat, 1987
along with the rodent framework residues at position 27, where the
amino acid is buried, and position 30 where the residue is predicted to be
solvent accessible near CDR1. In both cases no specific immune
response to initial treatments with the administered antibody was noted,
25 although responses to a second course of treatment was seen in one
study using CAMPATH-1H for the treatment of rheumatoid arthritis
(Frenken, 1991). There have been no reported clinical studies using
humanized antibodies in which other non-CDR solvent-accessible residues
have also been included in the design.

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The interactions of various cell surface proteins such as T cell
receptor/CD3 complex (TCR/CD3), MHC, CD8, ED45 and CD4 have been

-5-

shown to be important in the stimulation of T cell responses (Floury, 1991, Swartz, 1985, Strominger, 1980, Weiss, 1988). Two of these molecules, CD4 and CD3 have been found to be physically associated on the T cell (Saizawa, 1987, Anderson, 1988, Rojo, 1989, Mittler, 1989, 5 Dianzani, 1992). This association is critical to T cell receptor mediated signal transduction, in part due to their associated kinase and phosphates activities (Ledbetter, 1990). Molecules which can interrupt or prevent these interactions (i.e. antibodies) are currently recognized as therapeutically useful in the treatment of kidney allograft rejection (Ortho 10 Multicenter Transplant Group, 1985). A modification of antibody treatment, one in which several of the T cell surface proteins are directly bound together by one antibody might prove useful in current immunotherapy protocols. In addition to blocking cell adhesion or cell to cell interaction, antibodies which are capable of cross-linking several cell 15 surface proteins may result in stimulation of T cell activity or induction of aberrant signalling and thus produce modulation of the immune response (Ledbetter, 1990).

Bringing together molecules involved in T cell activation such as 20 CD3 and CD4, or CD3 and CD8, may be a potent method for immunoactivation. Previous studies have shown that cross-linking CD3 and CD4 with heteroconjugates composed of anti-CD3 and anti-CD4 antibodies result in a greater stimulation of Ca^{2+} flux than that observed with CD3 cross linked to itself or simultaneous cross-linking of CD3 and 25 CD4 by separate reagents (Ledbetter, 1990). Similarly, cross-linking CD3 and CD8 with immobilized antibody mixtures resulted in synergistic effects on T cell proliferation and IL-2 receptor expression (Emmrich, 1986 and 1987). These studies taken together point to a critical role for the interaction of CD3 with CD4/8 in T cell activation.

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The immunomodulatory effect of cross linking various T cell surface molecules can be both immunosuppressive and immunostimulatory.

-6-

Linkage of CD4 with itself or other T cell surface molecules has been shown to result in a different pattern of protein phosphorylation compared to cross-linking CD3 to itself (Ledbetter, 1990). This aberrant signalling may result as a consequence of binding both CD3 and CD4 simultaneously by a single cross-linking reagent. Previous studies have shown that pretreatment of T cells with antibody to cross-link CD4 to itself before anti-CD3 treatment inhibits T cell activation and promotes apoptosis (Newell, 1990). These results would argue that a reagent that crosslinks CD4 with CD3, or other T cell surface molecules, could be a potent immunosuppressant by virtue of inappropriate signalling through the TCR/CD3 complex.

Brief Summary of the Invention

In general, this invention contemplates the generation of anti-human CD3 mAbs with reduced activating properties as compared with OKT3. One way to achieve this is by transferring the complementary determining regions of OKT3 onto human IgG frameworks and then performing point mutations that reduce the affinity of the "humanized" anti-CD3 mAbs for FcR γ s. Studies show that whereas OKT3 and the parental humanized anti-CD3 mAbs activate T cells similarly, a humanized Fc variant fails to do so. Both the Fc variant and the activating anti-CD3 mAbs induce comparable modulation of the TCR and suppression of cytolytic T cell activity.

The invention further contemplates prolongation of human allograft survival with the nonactivating anti-CD3 mAbs, which retain significant immunosuppressive properties *in vivo*. Thus, the use of an Fc variant in clinical transplantation should result in fewer side effects than observed with OKT3, while maintaining its clinical efficacy.

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The present invention further contemplates the exploitation of an experimental model in which human splenocytes from cadaveric organ

-7-

donors are inoculated into severe combined immunodeficient mice (hu-SPL-SCID mice) to test the activating and immunosuppressive properties of these anti-human CD3 mAbs *in vivo*. Unlike injection of OKT3 or of the parental humanized mAb, administration of the Fc variant does not

5 result in T cell activation *in vivo*, as evidenced by the lack of induction of surface markers of activation, and of systemic human cytokines, including IL-2.

In accordance with long-standing patent law practice, the words

10 "a" and "an," when used to describe the invention in the specification or claims denotes "one or more" of the object being discussed.

Specific embodiments of the invention are as follows.

15 In one embodiment, the present invention contemplates a "humanized" version of the murine OKT3 antibody, a powerful immunosuppressive agent. In a preferred embodiment, the "humanized" monoclonal antibody of the present invention comprises a point mutation to leucine at position 234. In another embodiment, the antibody of the

20 present invention comprises a point mutation to glutamic acid at position 235.

Preferred embodiments of the present invention include anti-CD3 monoclonal antibodies that have reduced T cell activating properties

25 relative to murine OKT3. In some preferred embodiments, "humanized" murine OKT3 antibody having a human Fc region and a murine antigen binding region, form the basis for the production of the antibody. For example, the human Fc region can be an IgG1 or an IgG4 Fc portion. In some preferred antibodies, the human Fc region is an IgG1 portion.

30

In some embodiments the antibody has a mutated Fc receptor binding region, which leads to the antibody having reduced T cell

-8-

activating properties relative to murine OKT3. The Fc receptor binding region is found from about position 220 to about position 250 of the antibody, and mutations within this region are anticipated to have the potential to reduce the T cell activation properties of the antibodies by disrupting the region's ability to bind to Fc. The inventors have discovered that mutations in the region spanning about position 230 to about position 240 of the "humanized" antibodies can produce particular advantages. Comparisons of antibodies that bind to Fc those that do not bind to Fc suggest that changes in this region result in anti-CD3 antibodies that do not activate T cells. For example, some of the preferred antibodies comprise a mutation at position 234, at position 235, or at both. Anti-CD3 antibodies comprising one, two, three, four, five, or more mutations at one or more of positions 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, or 240, are expected to have advantages.

15

The purpose of the mutations is to disrupt the structure of the Fc receptor binding region. Therefore, while it is expected that mutations that insert an amino acid that differs significantly from the one that is deleted are most likely to disrupt the structure and have the desired effect, the invention is not limited to specific mutations at specific locations. For example, the inventors have had success by substituting charged amino acids such as glutamic acid for neutral amino acids such as leucine. The inventors have also had success inserting relatively general amino acids such as alanine for relatively complex amino acids such as phenylalanine. Those of skill in the art will understand the wide variety of mutations that can lead to the disruption of the region. For example, a neutral, positively, or negatively charged amino acid can be replaced with an amino acid of a different charge. Hydrophilic amino acids can replace hydrophobic amino acids, and vice versa. Large amino acids can replace small amino acids, and vice versa. A α -helix breaking, or other secondary structure disrupting, amino acid can be inserted.

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-9-

In one specific embodiment of the invention the "humanized" murine OKT3 antibody is gOKT3-5. For example, the inventors have found certain advantages for monoclonal antibodies made by placing a mutation from leucine to glutamic acid at position 235 of gOKT3-5. In
5 other specific embodiments, the "humanized" OKT3 antibody is gOKT3-7. For example, such gOKT3-7-based antibodies may comprise a mutation from phenylalanine to alanine at position 234, a mutation from leucine to alanine at position 235, or both. Certain preferred antibodies comprise a mutation from phenylalanine to alanine at position 234 and a second
10 mutation from leucine to alanine at position 235, with a specific example being Ala-Ala-IgG4.

Interestingly, the inventors have found that a gOKT3-7 antibody having an IgG1 Fc region and mutated to have alanine at both positions
15 234 and 235 (gOKT3-7(r₄-a/a) does not bind to complement. Specifically, this antibody does not bind to the C1q component and start the complement-mediated cascade. This result was totally unexpected and has the advantage of removing concerns about complement activation upon treatment with the antibodies. Those of skill will
20 understand the relative difficulties that complement activation could cause in human subjects.

Other embodiments of the invention include pharmaceutical compositions comprising the claimed anti-CD3 antibodies and a
25 physiologically acceptable carrier. The physiologically acceptable carrier can be any carrier that will allow the introduction of the claimed antibody in a therapeutic manner.

Other embodiments of the invention include methods of
30 suppressing immune response-triggered rejections of transplanted organ tissue. These methods comprise the step of administering to an organ transplant patient, either before, during or after transplantation, a

-10-

monoclonal antibody useful to modulate immunosuppressive activity. In certain preferred embodiments, the antibody is a "humanized" murine OKT3 monoclonal antibody that has a mutation. Other preferred methods for suppression of immune response-triggered rejection of transplanted organ tissue comprise the step of administering an antibody modulates immune response through binding to a first T-cell surface protein, designated CD3, and, simultaneously, to a second T-cell surface protein. For example, the second T-cell surface protein can be CD3, CD4, or CD8.

10 Brief Description of the Drawings.

The drawings and descriptions below form a portion of the specification.

15 Figure 1. Sequences of humanized OKT3 variable regions. Figures 1A and 1B show the alignments of the OKT3 light chain (1A) (SEQ ID NO: 6) and the heavy chain (1B) (SEQ ID NO: 10) variable domain amino acid sequence (row 1), the variable domain sequence from the human antibodies chosen as acceptor framework (row 2), and the humanized
20 OKT3 variable domain sequences (row's 3-5) (SEQ ID NOS: 8, 9, 12, 13 AND 14). The CDR choices are singly underlined. Rows 3-5 show only differences from the human acceptor sequence, with the non-CDR differences shown double underlined. Dashes indicate gaps introduced in the sequences to maximize the alignment. Numbering is as Kabat *et al.*,
25 (1987).

Figures 2A-2E. Amino acid and nucleotide sequence of murine OKT3.

Figure 3. Relative Affinity Determination. Competition of OKT3 and
30 humanized OKT3 antibodies for antigen against FITC-mOKT3. Increasing concentrations of unlabelled competitor antibody were added to a subsaturating concentration of FITC-mOKT3 tracer antibody, and were

-11-

incubated with human PBMC for 1 hour at 4° C. Cells were washed and fixed, and the amount of bound and free FITC-mOKT3 was calculated. The affinities of the antibodies were each calculated according to the formula $[X] - [mOKTK3] = (1/K_x) - (1/K_o)$, where K_o is the affinity of mOKT3, and K_x is the affinity of the competitor X. [] indicates the concentration of competitor at which bound/free tracer binding is $R_o/2$ and R_o is maximal tracer binding (Rao, 1992). A and B show results from separate experiments. solid squares: Orthomune @ OKT3; open circles: cOKT3(γ 4); closed triangles: gPLT3-1(γ 4); closed circles: gOKT3-5(γ 4); open squares: gOKT3-7(γ 4); open triangles: mOKT4A.

Figures 4A and 4B. Proliferation Assay. Proliferation of human PBMC to anti-CD3 antibody produced by COS cell transfection. PBMC were incubated for 68 hours in the presence of increasing amounts of anti-CD3 antibody, then pulsed with ^3H -thymidine for an additional 4 h, and the incorporation of ^3H -thymidine quantitated. closed squares: Orthomune@ OKT3; open squares: gOKT3-7(γ 4); open triangles: mOKT4A

Figure 5. OKT3 displacement assay. Serial dilutions of the "humanized" mAbs were used to competitively inhibit the binding of labeled OKT3 to the CD3 complex, as described in materials and methods. Values are expressed as a percent of the maximal fluorescence (arbitrary units attributed by the flow cytometer) achieved by binding of the labeled OKT3 alone. The symbols correspond to the following Abs: open circles, gOKT3-6 mAb; closed triangles, gOKT3-5 mAb; open squares, Leu-234 mAb; closed circles, Glu-235 mAb.

Figures 6A and 6B. FcR binding assay. A. Inhibition of binding of PE-coupled murine IgG2a to FcR on U937 cells by anti-CD3 mAbs. Different concentrations of the mAbs were added to the FcR-bearing U937 cell-line, previously stimulated with interferon- γ , to compete for the binding of a

-12-

PE-labeled IgG2a. The data are expressed as a percent of maximal fluorescence as described in Figure 1. B. Inhibition of ^{125}I -labelled human IgG binding to human FcR on U937 cells by murine and "humanized" OKT3. FcR binding activity to FcR on U937 cells was measured using a competitive inhibition assay as described in materials and methods. The results have been normalized so that the maximum binding of ^{125}I -hulgG in the absence of inhibitor equals 100%. In this experiment the maximum binding (2750 cpm) was 15% of the total radioactivity added. The symbols for both figures correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; open squares, Leu-234 mAb; closed circles, Glu-235 mAb.

Figure 7. N-terminal of CH₂ domain.

Figure 8. Mitogenicity induced by murine and "humanized" anti-CD3 mAbs. PBMC were incubated for 72 hours with serial dilutions of the mAbs before the addition of $1\mu\text{Ci}$ /well of H^3 Thymidine. Proliferation is depicted as the mean counts per minute (CPM) of triplicates (SEM < 10%). These data are representative of the proliferation obtained with PBMC with 3 different donors. The symbols correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

Figures 9A and 9B. Expression of markers of activation on the surface of T cells after stimulation with murine and "humanized" OKT3 mAbs. T cell expression of Leu 23 and IL-2 receptor was determined after culture of PBMC for 12 or 36 hours respectively, in the presence of varying concentrations of the anti-CD3 mAbs. The cells were stained with FITC-coupled anti-Leu 23 or anti-IL-2 receptor Abs and the fraction of T cells (CD2 or CD5-positive cells, counterstained by PE-coupled Abs) expressing the markers of activation were determined by FCM. The symbols

-13-

correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

Figure 10. Release of TNF induced by murine and "humanized" OKT3 mAbs. PBMC were cultured with serial dilutions of the different Abs for 24 hours. The concentration of TNF- α was determined by ELISA, using a commercial kit. Values are expressed as the mean of triplicates (SEM < 10%). The symbols correspond to the following Abs: open triangles, OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

Figures 11A-11C. Modulation and coating of the TCR achieved by the anti-CD3 mAbs. PBMC were incubated for 12 hours with various amounts of the anti-CD3 mAbs. Coating and modulation of the TCR complex was quantitated by FCM as explained in materials and methods. T cells were counterstained with PE-coupled anti-CD5 Ab. The bottom black boxes correspond to the total percentage of CD3 complexes that are modulated, the middle grey boxes to the percentage of CD3 complexes coated by the anti-CD3 mAbs and the upper white dotted boxes to the percentage of CD3 complexes uncoated on the surface of T lymphocytes.

Figure 12. Inhibition of T cell cytotoxic activity by "humanized" OKT3 mAbs. HLA A2-specific effector CTLs were generated by secondary mixed lymphocyte culture. Lysis of an A2-expressing LCL target was quantitated by a ^{51}Cr -release assay. Values are expressed as percent of maximum specific lysis. (Maximum specific lysis was determined to be 60% of the maximum lysis observed with 0.1 M HCL). Results represent the mean of triplicates (SEM < 10%). The symbols correspond to the following Abs: open circles, gOKT3-6 mAb; open triangles; OKT3; closed triangles, gOKT3-5 mAb; closed circles, Glu-235 mAb.

-14-

Figures 13A-13C. Variations of mean fluorescence of CD4 and CD8 surface markers induced by anti-CD3 mAbs.

Figure 14. CD4 binding to RES-KW3 cells.

5

Figure 15. CD4 binding on ELISA plates.

FIGURE 16. T cell proliferation to "humanized" mAbs. ^3H -thymidine incorporation by PBMC induced by soluble anti-CD3 mAbs was examined.

10 Human PBMCs were incubated with serial log dilutions of soluble OKT3 (closed circles), 209-IgG4 (closed squares), 209-IgG1 (closed triangles) or Ala-Ala-IgG4 (closed circles) mAbs for 72 hours, pulsed with ^3H -thymidine for an additional 4 hours, and quantified by using scintillation counting. All data is expressed as mean counts per minute
15 of triplicate samples.

FIGURE 17. Serum levels of anti-CD3 mAbs. Hu-SPL-SCID mice received OKT3, 209-IgG1 or Ala-Ala-IgG4 ($100\text{ }\mu\text{g}$ in 1 ml PBS ip). The animals were bled 1, 2 and 8 days after the injection. Serum levels of anti-CD3
20 were measured by FCM as described in materials and methods. Results are expressed as Mean \pm SEM of 5 animals per group.

FIGURE 18. Ala-Ala-IgG4 does not induce upregulation of CD69.

25 Hu-SPL-SCID mice were treated with PBS (1 ml) or OKT3, 209-IgG1 or Ala-Ala-IgG4 ($100\text{ }\mu\text{g}$ in 1 ml PBS ip). Spleens were harvested 24h after the injection, prepared into single cell suspensions and analyzed by FCM. The mean fluorescence obtained with anti-human CD69 on CD4^+ and CD8^+ human T cells of PBS-treated mice was used as baseline. Results are expressed as the percent increase from that baseline (Mean \pm SEM of
30 5 animals per group) and are representative of 4 independent experiments.

-15-

FIGURE 19. Production of human IL-2 after injection of anti-CD3 mAbs.

Hu-SPL-SCID mice received PBS (1 ml) or 145-2C11, OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μ g in 1 ml PBS ip). Mice were bled 2h after the injection, and sera were analyzed for human IL-2 levels, using a bioassay, as described in materials and methods. Results are displayed as the Mean \pm SEM of 4 mice/group, and are representative of 2 independent experiments.

FIGURE 20. Prolongation of human allograft survival by anti-CD3 mAbs.

SCID (4 mice) and hu-SPL- SCID mice (29 mice) were grafted with allogeneic human foreskin. Hu-SPL-SCID mice were treated with PBS (1 ml/d for 14 days, 4 mice), 145-2C11 (4 mice), OKT3 (8 mice), 209-IgG1 (6 mice) or Ala-Ala-IgG4 (5 mice). mAbs were administered ip at 50 μ g/day for 5 days followed by 10 μ g/day for 10 days. Results are representative of 3 independent experiments. A two-tailed FISHER EXACT test was used to compare the various groups in the 3 skin graft experiments performed. No difference in efficacy was found between the different Abs as the best results were achieved by different Abs in each experiment (OKT3 vs. 209-IgG: $p=0.12$; OKT3 vs Ala-Ala-IgG: $p=1.0$; 209-IgG vs. Ala-Ala-IgG: $p=0.23$).

Detailed Description of the Invention

I. The Invention.

25

The potent immunosuppressive agent OKT3 is a murine IgG2a mAb directed against the CD3 complex associated with the human TCR (Van Wauwe, 1980). However, the administration of OKT3 to transplant recipients induces the systematic release of several cytokines, including IL-2, IL-6, TNF- α and IFN- γ (Abramowicz, 1989; Chatenoud, 1989). This production of cytokines has been correlated with the adverse side-effects frequently observed after the first injection of OKT3 (Van Wauwe, 1980;

-16-

Chatenoud, 1989; Thistlethwaite, 1988), and may augment the production of anti-isotypic and anti-idiotypic antibodies occurring in some patents after one or two weeks of treatment, then can neutralize OKT3 and preclude subsequent treatments of rejection episodes (Thistlethwaite, 5 1988).

Several pieces of evidence strongly suggest that these side-effects are a consequence of the cross-linking between T lymphocytes and Fc receptor (FcR)-bearing cells through the Fc portion of OKT3, resulting in activation of both cell types (Debets, 1990; Krutman, 1990): 1.) anti-CD3 mAbs did not stimulate T cell proliferation *in vitro*, unless the Ab was immobilized to plastic or bound to FcR+ antigen presenting cells included in the culture (van Lier, 1989); 2.) the cross-linking of OKT3 through FcRs I and II enhanced proliferation in response to IL-2, *in vitro* 10 (van Lier, 1987); 3.) proliferation of murine T cells induced by 145-2C11, a hamster mAb directed against the murine CD3 complex, could be blocked by the anti-FcR Ab, 2.4G2; 4.) the injection into mice of F(ab')₂ fragments of 145-2C11 induced significant immunosuppression without triggering full T cell activation (Hirsch, 1990) and was less toxic in mice 15 than the whole mAb (Alegre, 1990); 5.) the administration of an OKT3 IgA switch variant that displayed a reduced FcR-mediated T cell activation as compared with OKT3 IgG2a, resulted in fewer side effects in chimpanzees *in vivo* (Parleviet, 1990). 20

25 Thus, theoretically, improvement of anti-CD3 mAb therapy can be obtained by molecularly modifying OKT3 to reduce its affinity for FcRs. The mutated Ab obtained would lead to lower cellular activation and acute toxicity *in vivo*, but conserved immunosuppressive properties.

II. The Immune System.

The immune system of both humans and animals include two
5 principal classes of lymphocytes: the thymus derived cells (T cells), and
the bone marrow derived cells (B cells). Mature T cells emerge from the
thymus and circulate between the tissues, lymphatics, and the
bloodstream. T cells exhibit immunological specificity and are directly
involved in cell-mediated immune responses (such as graft rejection). T
10 cells act against or in response to a variety of foreign structures
(antigens). In many instances these foreign antigens are expressed on
host cells as a result of infection. However, foreign antigens can also
come from the host having been altered by neoplasia or infection.
Although T cells do not themselves secrete antibodies, they are usually
15 required for antibody secretion by the second class of lymphocytes, B
cells.

A. T cells.

There are various subsets of T cells , which are generally defined
20 by antigenic determinants found on their cell surfaces, as well as
functional activity and foreign antigen recognition. Some subsets of T
cells, such as CD8⁺ cells, are killer/suppressor cells that play a regulating
function in the immune system, while others, such as CD4⁺ cells, serve
to promote inflammatory and humoral responses. (CD refers to cell
25 differentiation cluster; the accompanying numbers are provided in
accordance with terminology set forth by the International Workshops on
Leukocyte Differentiation, Immunology Today, 10:254 (1989). A general
reference for all aspects of the immune system may be found in Klein, J.
Immunology: The Science of Self-Nonself Discrimination, Wiley & Sons,
30 N.Y. (1982).

-18-

1. T cell activation.

Human peripheral T lymphocytes can be stimulated to undergo mitosis by a variety of agents including foreign antigens, monoclonal
5 antibodies and lectins such as phytohemagglutinin and concanavalin A. Although activation presumably occurs by binding of the mitogens to specific sites on cell membranes, the nature of these receptors, and their mechanism of activation, is not completely elucidated. Induction of proliferation is only one indication of T cell activation. Other indications
10 of activation, defined as alterations in the basal or resting state of the cell, include increased lymphokine production and cytotoxic cell activity.

T cell activation is an unexpectedly complex phenomenon that depends on the participation of a variety of cell surface molecules
15 expressed on the responding T cell population (Leo, 1987; Weiss, 1984). For example, the antigen-specific T cell receptor (TcR) is composed of a disulfide-linked heterodimer, containing two clonally distributed, integral membrane glycoprotein chains, α and β , or γ and δ , non-covalently associated with a complex of low molecular weight invariant proteins,
20 commonly designated as CD3 (the older terminology is T3) Leo, 1987).

The TcR α and β chains determine antigen specificities (Saito, 1987). The CD3 structures are thought to represent accessory molecules that may be the transducing elements of activation signals initiated upon
25 binding of the TcR $\alpha\beta$ to its ligand. There are both constant regions of the glycoprotein chains of TcR, and variable regions (polymorphisms). Polymorphic TcR variable regions define subsets of T cells, with distinct specificities. Unlike antibodies which recognize soluble whole foreign proteins as antigen, the TcR complex interacts with small peptidic antigen
30 presented in the context of major histocompatibility complex (MHC) proteins. The MHC proteins represent another highly polymorphic set of molecules randomly dispersed throughout the species. Thus, activation

-19-

usually requires the tripartite interaction of the TcR and foreign peptidic antigen bound to the major MHC proteins.

With regard to foreign antigen recognition by T cells the number of peptides that are present in sufficient quantities to bind both the polymorphic MHC and be recognized by a given T cell receptor, thus inducing immune response as a practical mechanism, is small. One of the major problems in clinical immunology is that the polymorphic antigens of the MHC impose severe restrictions on triggering an immune response. Another problem is that doses of an invading antigen may be too low to trigger an immune response. By the time the antigenic level rises, it may be too late for the immune system to save the organism.

The tremendous heterogeneity of the MHC proteins among individuals remains the most serious limiting factor in the clinical application of allograft transplantation. The ability to find two individuals whose MHC is identical is extremely rare. Thus, T cells from transplant recipients invariably recognize the donor organ as foreign. Attempts to suppress the alloreactivity by drugs or irradiation has resulted in severe side effects that limit their usefulness. Therefore, more recent experimental and clinical studies have involved the use of antibody therapy to alter immune function in vivo. The first successful attempt to develop a more selective immunosuppressive therapy in many was the use of polyclonal heterologous anti-lymphocyte antisera (ATG) (Starzl, 1967; Shield, 1979).

2. Antibody structure.

Antibodies comprise a large family of glycoproteins with common structural features. An antibody comprises of four polypeptides that form a three dimensional structure which resembles the letter Y. Typically, an antibody comprises of two different polypeptides, the heavy chain and the light chain.

-20-

An antibody molecule typically consists of three functional domains: the Fc, Fab, and antigen binding site. The Fc domain is located at the base of the Y. The arms of the Y comprise the Fab domains. The antigen binding site is located at the end of each arm of the Y.

5

There are five different types of heavy chain polypeptides which types are designated α , δ , ϵ , γ , and μ . There are two different types of light chain polypeptides designated k and λ . An antibody typically contains only one type of heavy chain and only one type of light chain, although any light chain can associate with any heavy chain.

10

Antibody molecules are categorized into five classes, IgG, IgM, IgA, IgE and IgD. An antibody molecule comprises one or more Y-units, each Y comprising two heavy chains and two light chains. For example IgG consists of a single Y-unit and has the formula $2k_2$ or $2\lambda_2$. IgM comprises of 5 Y-like units.

15

The amino terminal of each heavy light chain polypeptide is known as the constant (C) region. The carboxyl terminal of each heavy and light chain polypeptide is known as the variable (V) region. Within the variable regions of the chains are Hypervariable regions known as the complementarity determining region (CDR). The variable regions of one heavy chain and one light chain associate to form an antigen binding site. Each heavy chain and each light chain includes three CDRs. The six CDRs of an antigen binding site define the amino acid residues that form the actual binding site for the antigen. The variability of the CDRs account for the diversity of antigen recognition.

20

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B. Immune Response.

30

The principal function of the immune system is to protect animals from infectious organisms and from their toxic products. This system has evolved a powerful range of mechanisms to locate foreign cells, viruses,

-21-

or macromolecules; to neutralize these invaders; and to eliminate them from the body. This surveillance is performed by proteins and cells that circulate throughout the body. Many different mechanisms constitute this surveillance, and they can be divided into two broad categories --

5 nonadaptive and adaptive immunity.

Adaptive immunity is directed against specific molecules and is enhanced by re-exposure. Adaptive immunity is mediated by cells called *lymphocytes*, which synthesize cell-surface receptors or secrete proteins that bind specifically to foreign molecules. These secreted proteins are known as *antibodies*. Any molecule that can bind to an antibody is known as an *antigen*. When a molecule is used to induce an adaptive response it is called an *immunogen*. The terms "antigen" and "immunogen" are used to describe different properties of a molecule.

15 *Immunogenicity* is not an intrinsic property of any molecule, but is defined only by its ability to induce an adaptive response. *Antigenicity* also is not an intrinsic property of a molecule, but is defined by its ability to be bound by an antibody.

20 The term "immunoglobulin" is often used interchangeably with "antibody." Formally, an *antibody* is a molecule that binds to a known antigen, while *immunoglobulin* refers to this group of proteins irrespective of whether or not their binding target is known. This distinction is trivial and the terms are used interchangeably.

25

Many types of lymphocytes with different functions have been identified. Most of the cellular functions of the immune system can be described by grouping lymphocytes into three basic types -- B cells, cytotoxic T cells, and helper T cells. All three carry cell-surface receptors that can bind antigens. B cells secrete antibodies, and carry a modified form of the same antibody on their surface, where it acts as a receptor for antigens. Cytotoxic T cells lyse foreign or infected cells, and they

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-22-

bind to these target cells through their surface antigen receptor, known as the T-cell receptor. Helper T cells play a key regulatory role in controlling the response of B cells and cytotoxic T cells, and they also have T-cell receptors on their surface.

5

The immune system is challenged constantly by an enormous number of antigens. One of the key features of the immune system is that it can synthesize a vast repertoire of antibodies and cell-surface receptors, each with a different antigen binding site. The binding of the
10 antibodies and T-cell receptors to foreign molecules provides the molecular basis for the specificity of the immune response.

The specificity of the immune response is controlled by a simple mechanism -- one cell recognizes one antigen because all of the antigen
15 receptors on a single lymphocyte are identical. This is true for both T and B lymphocytes, even though the types of responses made by these cells are different.

All antigen receptors are glycoproteins found on the surface of
20 mature lymphocytes. Somatic recombination, mutation, and other mechanisms generate more than 10^7 different binding sites, and antigen specificity is maintained by processes that ensure that only one type of receptor is synthesized within any one cell. The production of antigen receptors occurs in the absence of antigen. Therefore, a diverse
25 repertoire of antigen receptors is available before antigen is seen.

Although they share similar structural features, the surface antibodies on B cells and the T-cell receptors found on T cells are encoded by separate gene families; their expression is cell-type specific.
30 The surface antibodies on B cells can bind to soluble antigens, while the T-cell receptors recognize antigens only when displayed on the surface of other cells.

-23-

When B-cell surface antibodies bind antigen, the B lymphocyte is activated to secrete antibody and is stimulated to proliferate. T cells respond in a similar fashion. This burst of cell division increases the number of antigen-specific lymphocytes, and this clonal expansion is the first step in the development of an effective immune response. As long as the antigen persists, the activation of lymphocytes continues, thus increasing the strength of the immune response. After the antigen has been eliminated, some cells from the expanded pools of antigen-specific lymphocytes remain in circulation. These cells are primed to respond to any subsequent exposure to the same antigen, providing the cellular basis for immunological memory.

In the first step in mounting an immune response the antigen is engulfed by an antigen presenting cell (APC). The APC degrades the antigen and pieces of the antigen are presented on the cell surface by a glycoprotein known as the major histocompatibility complex class II proteins (MHC II). Helper T-cells bind to the APC by recognizing the antigen and the class II protein. The protein on the T-cell which is responsible for recognizing the antigen and the class II protein is the T-cell receptor (TCR).

Once the T-cell binds to the APC, in response to Interleukin I and II (IL), helper T-cell proliferate exponentially. In a similar mechanism, B cells respond to an antigen and proliferate in the immune response.

The TCR acts in conjunction with a protein that is also expressed on the surface of the T-cell called CD3. The complex is the TCR-CD3 complex. Depending on the type of lymphocyte, the lymphocyte can also express other cell surface proteins which include CD2, CD4, CD8, and CD45. The interactions between these cell surface proteins are important in the stimulation of T cell response.

-24-

Two major sub-populations of T cells have been identified. CD4 lymphocytes can present on its cell surface, the CD4 protein, CD3 and its respective T cell receptor. CD8 lymphocytes can present on its cell surface, the CD8 protein, CD3 and its respective T cell receptor.

5

CD4 lymphocytes generally include the T-helper and T-delayed type hypersensitivity subsets. The CD4 protein typically interacts with Class II major histocompatibility complex. CD4 may function to increase the avidity between the T cell and its MHC class II APC or stimulator cell and enhance T cell proliferation.

10

CD8 lymphocytes are generally cytotoxic T-cells, whose function is to identify and kill foreign cells or host cells displaying foreign antigens. The CD8 protein typically interacts with Class I major histocompatibility complex.

15

C. Clinical use of antibodies.

Clinical trials of the ATG treatment suggested a significant reduction of early rejection episodes, improved long term survival and, most importantly, reversal of ongoing rejection episodes. However, the results were often inconsistent due to the inability to standardize individual preparations of antisera. In addition, the precise nature of the target antigens recognized by the polyclonal reagents could not be defined, thus making scientific analysis difficult. The advent of monoclonal antibody (mAb) technology provided the bases for developing potentially therapeutic reagents that react with specific cell surface antigens which are involved in T cell activation.

20

25

One of the clinically successful uses of monoclonal antibodies is to suppress the immune system, thus enhancing the efficacy of organ or tissue transplantation. U.S. Patent 4,658,019, describes a novel hybridoma (designated OKT3) which is capable of producing a monoclonal

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-25-

antibody against an antigen found on essentially all normal human peripheral T cells. This antibody is said to be monospecific for a single determinant on these T cells, and does not react with other normal peripheral blood lymphoid cells. The OKT3 mAb described in this patent
5 is currently employed to prevent renal transplant rejection (Goldstein, 1987).

One unexpected side effect of the OKT3 therapy was the profound mitogenic effect of the mAb in vivo (Ellenhorn, 1988).

10

In addition, other cell surface molecules have been identified that can activate T cell function, but are not necessarily part of the T cell surface receptor complex. Monoclonal antibodies against Thy-1, TAP, Ly-6, CD2, or CD28 molecules can activate T cells in the absence of foreign
15 antigen in vitro (Leo, 1989; Takada, 1984). Moreover, certain bacterial proteins although differing in structure from mAbs, also have been shown to bind to subsets of T cells and activate them in vitro (White, 1989).

The possibility of *selectively* down-regulating the host's immune
20 response to a given antigen represents one of the most formidable challenges of modern immunology in relation to the development of new therapies for IgE-mediated allergies, autoimmune diseases and the prevention of immune rejection of organ transplants. Similar considerations apply to an increasing number of promising therapeutic
25 modalities for a broad spectrum of diseases, which would involve the use of foreign biologically active agents potentially capable of modulating the immune response, provided they were not also immunogenic. Among these agents, one may cite (1) xenogeneic monoclonal or polyclonal antibodies (collectively referred to here as xlg) against different epitopes
30 of the patients' CD4⁺ cells (Cruse, 1989; Diamantstein 1986), administered alone or in combination with immunosuppressive drugs for the treatment of rheumatoid arthritis and other autoimmune diseases, or

-26-

for the suppression of graft-versus-host reactions and the immune rejection of organ transplants (Cruse, 1989).

5 The therapeutic effectiveness of these immunological strategies is undermined by the patients' antibodies which prevent these bullets from reaching their target cells. In addition, the repeated administration of these agents may result in serious complications, viz. serum sickness, anaphylactic symptoms (*i.e.* bronchospasm, dyspnea and hypotension) and/or the deposition in the liver of toxic immune complexes leading
10 frequently to hepatotoxicity.

D. Preparation of monoclonal and polyclonal antibodies.

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen, and collecting antisera from that immunized animal.
15 A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

20

As is well known in the art, a given polypeptide or polynucleotide may vary in its immunogenicity. It is often necessary therefore to couple the immunogen with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other
25 albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

Means for conjugating a polypeptide or a polynucleotide to a carrier protein are well known in the art and include glutaraldehyde, m-
30 maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

-27-

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's
5 adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies *inter alia*, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to
10 administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is
15 obtained, the immunized animal can be bled and the serum isolated and stored.

A monoclonal antibody of the present invention can be readily prepared through use of well-known techniques such as those exemplified in U.S. Pat. No 4,196,265, herein incorporated by reference. Typically, a
20 technique involves first immunizing a suitable animal with a selected antigen (e.g., a polypeptide or polynucleotide of the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized
25 animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells
30 are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are

-28-

aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media is supplemented with hypoxanthine.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce a monoclonal antibody, mice are injected intraperitoneally with between about 1-200 μg of an antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*). At some time (*e.g.*, at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant.

A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

-29-

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus denominated immortal. Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

10 Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are *hybridoma* cells. Like
15 myeloma cells, hybridoma cells grow indefinitely in culture.

Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes
20 necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media.

25 Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas.
30 The hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then

-30-

cultured in large amounts to produce an antibody of the present invention in convenient quantity.

5 III. Immunosuppressive modulation
 through use of "humanized" mAbs.

 In order to improve the effectiveness and expand the uses of OKT3, humanized versions of the antibody have been generated. It has been shown (Woodle, 1992) that simple transfer of the loop regions and the complementarity determining regions (CDR's) (Kabat, 1987), which
10 are believed to contain the antigen contacting amino acids, into a human framework was not sufficient in the case of OKT3 to provide the structure required for efficient antigen binding. Examination of the remaining framework residues identified several which could potentially contribute to a reconstitution of binding in a human framework. When amino acids
15 at these positions in the human framework were replaced with those from OKT3 to give gOKT3-5, antigen binding was shown to be fully restored. Subsequently, it has been noted (Jolliffe, 1991) that a number of these amino acids derived from the OKT3 sequence are not required to achieve a humanized antibody with the same affinity as murine OKT3.

20

 To reduce the immune responses observed in patients treated with murine OKT3, a "humanized" OKT3 (gOKT3-5), comprised of the complementary determining regions (CDR) of the murine anti-CD3 mAb and of the variable framework and constant regions of a human IgG4,
25 was developed. However, as a therapeutic drug, an additional problem associated with OKT3, the first-dose reactions attributed to the T cell activation by the mAb, remained. Since gOKT3-5 produces, *in vitro*, similar activation to OKT3, it is quite likely that the same side-effects might also occur with this drug *in vivo*. F(ab')₂ fragments of OKT3 have
30 led to potent immunosuppression and TCR modulation, *in vitro*. Non-activating F(ab')₂ fragments of anti-CD3 mAbs to mice was as efficacious as whole anti-CD3 in delaying skin graft rejection, while the F(ab')₂

-31-

fragments exhibited significantly reduced T cell activation and fewer side-effects in mice. However, the production of F(ab')₂ fragments in large quantities remains difficult. Furthermore, the half-life of this drug in the blood stream is relatively short, as compared with whole mAb. Thus, frequent injections of the F(ab')₂ fragments of anti-CD3 were necessary to achieve maximal immunosuppression, making the use of this mAb fragment inappropriate for clinical transplantation. Finally, recent studies have shown that even a small contaminant of whole mAb in the F(ab')₂ preparation ($< 1/10^4$ molecules) has a synergistic effect on T cell activation.

A. Point mutations in "humanized" mAbs.

The Fc portion of the murine IgG2a Abs, including OKT3, binds preferentially to the high affinity 72 kD FcR I (CD64) present on human macrophages and IFN- γ -stimulated polymorphonuclear leukocytes (Anderson, 1986; Lynch, 1990; Shen, 1987), but also to the low affinity 40 kD FcR II (CD32) that is found on human macrophages, β cells and polymorphonuclear neutrophils (Anderson, 1986; Petroni, 1988; Bentin, 1991). The CH2 region in the Fc portion of IgGs has been found to be the domain that selectively binds FcR I and II (Ollo, 1983; Woof, 1984; Burton, 1985; Partridge, 1986; Duncan, 1988). In fact, the exact binding segment has been localized to an area corresponding to amino acids 234 to 238 (Duncan, 1988) and the respective affinity of several isotypes has been determined (Gergely, 1990). Duncan *et al.* have shown that the mutation of a single amino acid in the FcR binding segment of a murine IgG2b, converting the sequence to that found in a murine IgG2a, resulted in a 100-fold enhancement of the binding to FcR (1988). Based on those data, a mutation was introduced into the Fc region of an anti-CD3 human IgG4 antibody resulting in a sequence similar to the low affinity sequence of the murine IgG2b. This mAb contains a glutamic acid rather than a leucine at position 235 of the human IgG4 heavy chain (Glu-235 mAb). The mutational analysis was performed on a "humanized" anti-CD3 mAb,

-32-

the gOKT3-5 mAb by splicing the murine complementarily determining regions into the human IgG4 framework gene sequence. The gOKT3-5 mAb was previously shown to retain binding affinity for the CD3 complex similar to murine OKT3 and all the *in vitro* activation and

5 immunosuppressive properties of OKT3. In addition, the gOKT3-5 mAb had an FcR binding sequence differing by only two amino acids from the same region on the murine IgG2b or by one amino acid in the murine IgG2a/human IgG1. Since a mutation in the FcR binding region of the mAb could modify the conformation of the molecule and thus be

10 responsible for a decrease in FcR binding regardless of the amino acid sequence obtained, we performed a control mutation of amino acid 234 from a phenylalanine into a leucine in order to mimic the FcR binding area found in the high affinity murine IgG2a and human IgG1. This mAb was designated Leu-234.

15

Therefore, the site-specific mutations described above were introduced into the Fc portion of the gOKT3-5 mAb to affect the binding of the Ab to FcR. The appropriate mutant of the anti-CD3 mAb was designed to exhibit the low-activating properties of F(ab')₂ fragments, the

20 purity of a monoclonal antibody and an increased serum half-life as compared with F(ab')₂ fragments or possibly even with murine OKT3, since chimeric mouse/human antibodies have been shown to circulate longer their murine counterpart. The resulting mAb thus avoids the acute toxicity and the immunization induced by OKT3, *in vivo*, although,

25 theoretically, the substitution of glutamic acid at position 235 in order to mimic murine IgG2b could also create an immunogenic epitope in the constant region of the humanized antibody.

In fact, a single amino acid substitution of a glutamic acid for a

30 leucine at position 235 in the Fc portion of the gOKT3-5 mAb resulted in a mAb which bound U937 cells 100-fold less than the murine OKT3. This mutation, which generated an FcR binding sequence similar to the

-33-

one found in murine IgG2b, resulted in a mAb with a 10-fold lower affinity for FcR than the murine IgG2b (data not shown). The reason for this difference is unclear but may imply that the interaction of the five amino acid-FcR binding region with the adjacent amino acids, which in the case of the Glu mAb are part of a human IgG4, is relevant to FcR binding.

All the Abs tested showed some modulation of the TCR after a culture of 12 hours. However, the Glu-235 mAb had to be added in higher concentrations or for a longer period of time to achieve maximal modulation. This suggests that low FcR binding might delay the induction of TCR internalization. All the Abs also inhibited CTL activity, indicating similar suppressive properties by this assay. Thus, altering the binding of the gOKT3-5 mAb by site-directed mutagenesis did not significantly affect the immunosuppressive ability of the mAb, *in vitro*.

The reduced binding of the Glu-235 mAb correlated with a marked decrease in the T cell activation induced by this Ab, as assessed by the absence of T cell proliferation, the decreased expression of cell surface markers of activation, the diminished release of TNF- α and GM-CSF and the lack of secretion of IFN- γ . The magnitude of T cell mitogenesis is known to correlate with the affinity of anti-CD3 mAbs for FcR I, whose relative binding is IgG1 = IgG3 > IgG4 for human subclasses of Abs and IgG2a = IgG3 > IgG1 > IgG2b for murine isotypes. The anti-CD3 mAbs employed in this study displayed an FcR binding as expected, with the human IgG4 gOKT3-5 mAb binding less avidly to U937 cells than murine IgG2a OKT3 or Leu-234 mAb, but with much higher affinity than the Glu-235 mAb.

The activation induced by the different anti-CD3 mAbs tested did not entirely correlate with their affinity for FcRs. In spite of the increased affinity of OKT3 for FcRs as compared with the gOKT3-5 mAb, no significant difference in the T cell activation was observed between the

-34-

two mAbs. One explanation could be that activation is maximal whenever a certain threshold of cross-linking between T lymphocytes and FcR is attained. Another possibility is that the binding of the mAb to the CD3 antigen potentiates its avidity for FcR-bearing cells.

5

The extent of the functional changes generated in the FcR binding region of the gOKT3-5 mAb that form the Glu-235 mAb has further implications. The ability of certain isotypes of anti-CD3 mAbs to activate T cells and mediate ADCC has been shown to vary in the population.

10 Murine IgG2a and IgG3 anti-CD3 mAbs are mitogenic for virtually all individuals. In contrast, murine IgG1 and IgG2b mAbs induce proliferation in only 70% and 5% to 10%, respectively. The Glu mAb, which appears to function as a non-activator IgG2b in a small fraction of the population. However, even in these individuals, IgG2b mAbs seem to trigger a
15 different pathway of activation. For instance, in contrast to other anti-CD3 isotypes, IgG2b mAbs do not induce the production of IL-2 or IFN- γ . Thus, the proliferation observed in the small subset of the patient population may be an IL-2 independent T cell mitogenesis, which has previously been reported in other settings. More importantly, the reduced
20 FcR binding of the Glu-235 mAb to FcR, as compared with murine IgG2b Abs, may be sufficient to abrogate the activation of even this subset of individuals.

In one embodiment, the present invention contemplates a class of
25 homo-bifunctional antibodies, a humanized version of OKT3 which also interacts with CD4. This humanized antibody has an Fv region containing the CD3 ϵ antigen specificity of OKT3 and an Fc region from either human IgG1 or IgG4 antibody. The humanized anti CD3 antibody binds CD4 directly, either immobilized on plastic or on CD4⁺, CD3⁺, FcR cells. Initial
30 mapping experiments suggest that the binding occurs near the OKT4A epitope on CD4. The weak interaction of some antibodies (but not human IgG4) with this region of CD4, independent of antigen/antibody binding

-35-

site, has been reported (Lanert, 1991). However, unlike these reports, the antibody of the present invention binds with either a $\gamma 1$ or a $\gamma 4$ heavy chain. The CD4 binding site on humanized OKT3 has been mapped to the Fab fragment and probably resides in the framework sequences of the variable region.

By use of a monoclonal antibody of the present invention, specific polypeptides and polynucleotides of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides and polynucleotides can be isolated and purified by techniques such as *antibody-affinity chromatography*. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide or polynucleotide is then easily removed from the substrate and purified.

VII. Pharmaceutical Compositions.

In a preferred embodiment, the present invention provides pharmaceutical compositions comprising antibodies immunoreactive with CD3 and CD4 cell surface antigens.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes intravenous, intramuscular, intraarterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspen-

-36-

sion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed
5 are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

10

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminant, such as defective interfering adenovirus particles or endotoxins and other
15 pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

20 A carrier can also be a liposome. Means for using liposomes as delivery vehicles are well known in the art [See, *e.g.*, Gabizon *et al.*, 1990; Ferruti *et al.*, 1986; and Ranade, V.V., 1989].

A transfected cell can also serve as a carrier. By way of example,
25 a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (*e.g.* injected intravascularly).

30 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow

-37-

represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

10

EXAMPLE 1 Mutation in the Fc portion of the human-OKT3 mAb.

Mutations of the phenylalanine in position 234 into a leucine to increase the affinity of the binding of the mAb to FcR I (Leu-234), or of the contiguous leucine (235) into a glutamic acid to reduce FcR binding (Glu-235) were performed as follows: ultracompetent CJ 236 E. coli (Invitrogen, San Diego, CA) were transformed with pSG5 containing the heavy chain gene of the gOKT3 mAb. The bacteria were allowed to grow in LB broth supplemented with uridine (25 mg/mL), ampicillin (100 μ g/mL) until reaching an optical density of 0.35 at a wave length of 600 nm.

The CJ 236 E. coli were infected with helper phage M-13 (pfu) (Stratagen) to generate uridine incorporated single stranded template. An oligonucleotide synthesized with thymidine and containing the desired mutation was then annealed to the uridine-single-stranded template to serve as a primer for the replication of the plasmid after the addition of deoxynucleotides, T7 polymerase and T4 ligase; the wild type DNA thus contains uridine, while the mutated plasmid obtained utilizes thymidine. The synthesis reaction was stopped with EDTA 0.5M and Tris HCl-EDTA 1M, and 10 μ l were transformed into competent DH5 E. coli that degrade uridine-DNA and thus grew on ampicillin-selected media when transformed with the mutated construct. The plasmid was isolated by Qiagen minipreps; the mutated sequence in pSG5 was co-introduced

-38-

with the psG5 vector containing the light chain of the mAb into COS-1 cells for transient expression of the mutant immunoglobulin.

5 **EXAMPLE 2** Generation and identification of
 OKT3 variable region sequences.

OKT3 variable region sequences were derived from oligo-dT primed cDNA from OKT3 hybridoma cells using the Amersham International Plc. cDNA synthesis kit. The cDNA was cloned in pSP64 using EcoR1 linkers. *E. coli* clones containing light and heavy chain cDNAs were identified by
10 oligonucleotide screening of bacterial colonies using the oligonucleotides:
5' TCCAGATGTTAAGTCTCAC (SEQ ID NO: 15) for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC (SEQ ID NO: 16) for the heavy chain, which is complementary to a sequence in the mouse
15 IgG2a constant CH1 domain region.

The amino acid sequences for the variable regions deduced from the sequences of the cDNAs are shown in FIG. 1A (row 1) for the light chain and FIG. 1B (row 1) for the heavy chain. The CDR's are shown
20 with the single underlining. The light chain is a member of the mouse V_L subgroup VI and uses a $J_{\kappa}4$ minigene. The heavy chain is probably a member of the mouse V_H subgroup II, most probably IIb, although it also has significant homology to the consensus for group Va. The D region is currently unclassified and the J_H region is J_H2 . In terms of the loop
25 predictions for the hypervariable regions proposed by Chothia et al., 1987, the loops can be assigned to canonical structures 1 for L1, 2 for L2 and 1 for L3, and to canonical structures 1 for H1 and 2 for H2, Chothia et al., have not yet predicted canonical forms for H3.

30 The light chain variable region amino acid sequence shows a high degree of homology to the Ox-1 germline gene and to the published antibodies 45.2.21.1, 14.6b.1 and 26.4.1 (Sikder, 1985). The heavy

-39-

chain variable region amino acid sequence shows reasonable homology to a subgroup of the J558 family including 14.6b.1. Some antibodies with these combinations of light and heavy chain genes have previously been shown to have affinity for alpha-1-6 dextran.

5

EXAMPLE 3 Design and construction of humanized OKT3 genes.

The variable region domains for the humanized antibodies were designed with mouse variable region optimal codon usage (Grantham, 1986) and used the signal sequences of the light and heavy chains of mAb B72.3 (Whittle, 1987). Immediately 5' to the initiator ATG a 9bp Kozak sequence (Kozak, 1987), GCCGCCACC (SEQ ID NO: 17), was inserted. 5' and 3' terminal restriction sites were added so that the variable regions could be attached directly to the DNA sequences for the human IgG4 and Kappa constant regions prior to cloning into the eukaryotic expression vectors.

15

The variable regions were built either by simultaneously replacing all of the CDR and loop regions by oligonucleotide directed, site-specific mutagenesis (Olo, 1983) of a previously constructed humanized variable region for B72.3 cloned in M13 (Emtage *et al.*), or by assembling the sequence using synthetic oligonucleotides ranging in size from 27-67 base pairs and with 6 base overhangs. The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA Synthesizer and purified by HPLC. The oligonucleotides were enzymatically phosphorylated, paired, annealed and then equimolar aliquots of each pair were mixed and ligated. The cloning sites were exposed by restriction digestion of the ligation mixture and the correctly sized fragments were identified and cloned directly into the expression vectors, 5' to the constant regions, prior to sequencing and expression.

20

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For the design of the humanized OKT3 variable region sequences, REI (Kabat, 1987) was chosen as the human light chain framework, and

-40-

KOL was chosen for heavy chain variable region. In both cases antibodies were selected for which a structure had been determined by X-ray crystallography so that a structural examination of individual residues in the human variable region frameworks could be made. The variable
5 region sequences of the human acceptor frameworks are shown in FIG. 1A and 1B (row 2) (SEQ ID. NOS: 7 and 11).

For comparison purposes, the amino acid and nucleotide sequences for murine OKT3 (SEQ ID NOS: 2-5 and 1), as obtained from *Sequences of Proteins of Immunobiological Interest 4/e* (1987), are provided in FIG. 2.
10

Row 3 in each of FIG. 1A (SEQ ID NO: 8) and 1B (SEQ ID NO: 12) shows the sequences for the variable regions of the initial design, gL and gH. Only differences from the human acceptor sequence are shown. For
15 gL the CDR choices were as suggested by Kabat et al., and no other non-CDR murine residues were used. For gH the OKT3 CDR's, as suggested by reference to Kabat et al., were substituted into the KOL sequence along with the murine residues at positions 27, 28 and 30 which are normally bound in a loop region adjacent to CDR1 (Chothia,
20 1987; 1989). The importance of residue 27 as a determiner of antigen binding was shown by Riechmann et al., (Reichman, 1988) in the reconstitution of binding activity of the CAMPATH-1 antibody. The residues 28 and 30 are predicted to be at the surface of the antibody and near to CDR1. Residue 29 is the same in both KOL and OKT3 (Figure 1B)
25 and therefore does not require to be altered.

The DNA sequences coding for the initial humanized light and heavy variable regions were constructed by simultaneous replacement through site-directed mutagenesis of sequences in previously generated
30 light and heavy chain DNAs of a humanized form of antibody B72.3. The DNA sequences coding for the humanized variable regions were then attached to the human gamma-4 and kappa constant region sequences

-41-

and inserted into expression vectors as described for the chimeric genes. The gL and gH genes, when co-expressed in COS cells yield antibody gOKT3-1.

5 gOKT3-1 binds poorly to HPB-ALL cells and is not able to block the binding of mOKT3 to the cells (FIG. 3). Therefore it was clear that further OKT3 residues outside of the CDRs needed to be considered for substitution into the humanized antibody. For the light chain these positions are at 1 and 3 which by reference to known structures for
10 antibody variable regions are probable surface residues located near to the CDR's, residue 46 which is usually at the domain interface and the packing residue at 47, gLA has all four residues derived from the murine sequence while gLC has murine residues at positions 46 and 47 only.

15 Similarly, for the heavy chain, a number of locations were considered. These were at positions 23, 73 and 76 which are believed, by analogy with known antibody structures, to be partly or completely solvent exposed residues near the CDRs; at positions 6, 24, 48, 49, 71, 78 and 88 which are residues believed either to be involved in positioning
20 of the CDRs and/or in intradomain packing, and the variable domain interface residue 91. Finally at residue 63 in CDR2, which is usually an intra-domain packing residue, the residue found in KOL was used so that potentially unfavorable contacts with other packing residues from the human framework could be avoided. A number of light and heavy chain
25 variants were built to assess the contribution of these framework residues. It was found by experiment that residues 1 and 3 on the light chain were not required to be derived from the murine sequence, but that one or both of residues 46 and 47 should be derived from the murine sequence. FIG. 1A, row 4 (SEQ ID NO: 9) shows the sequence of gLC
30 which differs from gL by having the murine sequences at residues 46 and 47. Similarly, in the heavy chain it was found that while incorporating all of the modifications described above to give gHA (FIG. 1B row 4) (SEQ ID

-42-

NO: 13), and co-expressing this gene with cL or gLC would lead to antigen binding equivalent to cOKT3 or mOKT3, some of the residues were not necessary to retain equivalent binding affinity. In particular it was found when the KOL sequences were used at positions 71, 73, 76,
5 88 and 91 in the gHG gene, co-expression of gHG with cL or gLC led to antigen binding equivalent to cOKT3 or mOKT3. Therefore, the binding affinity of the gLC/gHA(gOKT3-5) and gLC/gHG(gLC/gHG) combinations have been analyzed in more detail.

10 Large scale COS cell expression preparations were made and the humanized antibody was affinity purified by Protein A. Relative binding affinities were measured. FIG. 3 shows results from two such experiments. The affinity of mOKT3 for antigen (K_a) was measured to be $1.2 \times 10^9 \text{ M}^{-1}$ by Scatchard analysis. This value for mOKT3 compares
15 well to that of $1.3 \times 10^9 \text{ M}^{-1}$ by Scatchard analysis. This value for mOKT3 compares well to that of $1.3 \times 10^9 \text{ M}^{-1}$ determined previously (Gergely, 1990). In FIG. 3A, gOKTE-5 was compared with cOKT3 and mOKT3 for competition against mOKT3. Values of $1.2 \times 10^9 \text{ M}^{-1}$ and $1.1 \times 10^9 \text{ M}^{-1}$ 2343 obtained for the cOKT3 and gOKT3-5 antibodies
20 respectively.

Subsequently, (FIG. 3B) similar results were obtained for gOKT3-7 (K_a $1.4 \times 10^9 \text{ M}^{-1}$) compared to $1.2 \times 10^9 \text{ M}^{-1}$ for mOKT3, $1.4 \times 10^9 \text{ M}^{-1}$ for cOKT3 and $1.1 \times 10^9 \text{ M}^{-1}$ for gOKT3-5. These experiments show that
25 the antigen binding activity of OKT3 has been successfully transferred to the humanized antibodies.

Previous studies have indicated that mitogenic potency is a sensitive parameter of the T cell activation properties of anti-CD3 mAbs (Woodle, 1991). In an earlier study it was shown that gOKT3-5 still demonstrated
30 mitogenic potency even in the context of an IgG4 isotype. Therefore, the activation potency of gOKT3-7 antibody was assessed by quantitating proliferating responses. gOKTE-7 demonstrated mitogenic potency

-43-

equivalent to that of mOKT3 (FIG. 4). This suggests that cross-linking of the bound antibody still occurs with the $\gamma 4$ isotype leading to proliferative signals. A therapeutic humanized OKT3 antibody may need further alterations to the constant region to minimize such effects.

5

EXAMPLE 4 Construction and expression of chimeric OKT3 genes.

The murine cDNAs were assembled into expression vector controls for the biological function of the humanized antibodies. The murine variable region cDNA sequences were attached to human k light chain and $\gamma 4$ heavy chain constant region DNA sequences following a
10 previously described strategy to generate chimeric OKT3 (cOKT3) genes which were then inserted into eukaryotic expression vectors. As the ultimate aim is to design a humanized OKT3 iGg antibody which can efficiently bind to CD3 while retaining useful effector pharmacokinetics and have no first dose side effects, a reduced affinity for FcR was built
15 into the constructs by using the $\gamma 4$ gene.

Small scale COS cell expression and metabolic labelling studies were as described (Whittle, 1987). Large scale COS cell expression
20 studies were performed in roller bottles, harvesting the product supernatant 5 days after transfection. (T. Livelli, Specialty Media Inc., Lavallette, New Jersey). Material from large scale transfections was purified by Protein A Sepharose chromatography. The yield of assembled antibody in COS cell supernatants was measured as described by Woodle
25 et al., 1992.

Murine OKT3, cOKT3, and murine/chimeric hybrid antibodies expressed from COS cells were shown to bind to antigen equivalently to mOKT3 and to block the binding of MOKT3 to CD3 positive cells.

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-44-

EXAMPLE 5 Transient expression of murine
and human-OKT3 mAbs genes.

COS-1 cell expression studies were performed using reagents and
5 procedures from a transient expression kit (Specialty media, Lavallette,
NJ) modified for use in roller bottles (T. Livelli, Specialty Media, personal
communication). Product supernatants for purification of the test Abs
were harvested 6 days after transfection.

10 ELISA assays were performed to determine the yield of assembled
"humanized" antibody in COS cells supernatants. Ninety-six well plates
were coated with F(ab')₂ goat anti-human Fc antibody. COS cell
supernatants were added and incubated for one hour at room temperature
and washed. Horseradish peroxidase-conjugated goat anti-human kappa
15 chain (Caltag) was used with o-phenylenediamine (OPD) for detection.
Purified human IgG was used as standard.

EXAMPLE 6 Mutated "humanized" OKT3 mAbs bind
to the CD3 complex of T cells with
20 the same affinity as murine OKT3.

The Fc portion of the gOKT3-5 mAb was mutated according to
procedures described above in order to alter its binding to FcR-bearing
cells. A phenylalanine was substituted for a leucine in position 234 (Leu-
234), or the adjacent leucine (235) was transformed into a glutamic acid
25 (Glu-235). The affinity of the gOKT3-5 mAb for the TCR complex was
previously shown to be similar to that of OKT3 (Van Wauwe, *et al.*,
1980). Although changes in the Fc portion of the mAb should not alter
Ag binding affinity, it was important to show that point mutations in the
CH2 region of the Ab, close to the hinge, did not impair the binding of the
30 Leu-234 and the Glu-235 mAbs to the CD3 antigen.

A displacement assay was performed to examine the ability of the
mutated Abs to competitively inhibit the binding of murine OKT3 to

-45-

human T cells. Human peripheral blood acute lymphocytic leukemia cells were re-suspended in flow cytofluorimetry (FCM) buffer at 5×10^5 cells/mL. Dilutions of the anti-CD3 mAbs were added and incubated at 4°C for 1 hour. Fluorescein isothiocyanate (FITC) was dissolved in N,N-dimethyl formamide (DMF) to give a 10 mg/ml solution. FITC/DMF was added to purified mAb at 1:10 w/w and incubated at 25°C for four hours, followed by dialysis into PBS containing an anion exchange resin (AG1-X8, 200-400 mesh, chloride form; Bio-Rad). Aggregates were removed prior to use by airfuge centrifugation (Becton-Dickinson). A fixed saturating amount of OKT3-FITC was added, and the cells were further incubated for 1 hour at 4°C, washed and analyzed by flow cytofluorimetry (FCM).

One or two-color FCM were performed using a FACScan flow cytometer, interfaced to a Hewlett-Packard 310 computer. Data analysis were performed using Consort-30 software. Logarithmically amplified fluorescence data were collected on 10,000 viable cells, as determined by forward and right angle light scatter intensity. One-color fluorescence data were displayed in histogram mode with fluorescence intensity on the x axis and cell number of the y axis. Two-color fluorescence data were displayed as contour plots with green (FITC) fluorescence on the x axis and orange (phycoerythrin) fluorescence on the y axis. All FCM staining procedures were performed at 4°C in FCM buffer.

The results of this assay are shown in FIG. 5. The data is presented as % inhibition of maximal fluorescence intensity (determined by OKT3-FITC binding in the absence of blocking Ab). Both mutant Abs displayed a similar affinity for their epitope as the parental gOKT3-5 mAb. In contrast, the gOKT3-6 mAb, a different "humanized" OKT3 which has a very weak binding activity for the CD3 antigen (Van Wauwe, *et al.*, 1980), was unable to displace the OKT3 mAb. These results correlate with the data obtained previously on a panel of isotype-switch variants of

-46-

murine anti-CD3 mAbs. In those studies, the anti-CD3 mAbs expressing different isotypes had a comparable avidity for the TCR complex as assessed by Scatchard analysis (Van Wauwe, *et al.*, 1980), or by precipitation of the TCR complex and cross-blocking experiments. Thus, any differences in the activation or suppressive properties of the mutated Abs could not be attributed to a modified affinity of the combining site of the anti-CD3 mAbs for T cells.

EXAMPLE 7 Binding of the mutant anti-CD3
mAbs to FcR on U937 cells.

The mutations generated in the CH2 region of the human IgG4 gOKT3-5 either mimicked the amino acid sequence of the FcR binding region of a human IgG1 (Leu-234), which has a higher affinity for human FcR I than human IgG4, or of a murine IgG2b (Glu-235) that binds weakly to FcR I but still binds to human FcR II. In order to determine the effects of those mutations on FcR binding, the FcR binding affinity of the various "humanized" OKT3 mAbs were tested on the monocytic U937 cell line that bears FcR I and II by displacement of either a PE-coupled murine IgG2a (FIG. 6A) or of a ¹²⁵I-labelled human IgG1 (FIG. 6B).

The murine anti-CD5 IgG2a-PE, OKT3E IgG2b, OKT3D IgG2b, OKT3 IgG2a, and a human IgG4 Ab FITC-coupled as described *supra*, were used to compete for binding in the FcR binding assay. Phycoerythrin-coupled (PE) anti-CD2 and anti-CD5 used as counterstains in the activation assays were purchased from Coulter Immunology. Modulation and coating of the TCR were determined using FITC-coupled OKT3 IgG2a and OKT3D IgG2a as described below.

FcR binding assays were performed using the FcR I- and II-bearing U937 human cell line.

-47-

For competitive inhibition assay with PE-coupled murine anti-CD5 IgG2a, 30×10^6 cells were cultured overnight at 37°C in complete media in the presence of 500 U/mL of human IFN- γ to enhance the expression of FcR I. The cells were washed three times with DMEM containing 25 μM HEPES, incubated for 2 hours at 37°C in FCS-free media and washed twice in DMEM and once in flow cytofluorimetry (FCM) buffer (PBS containing 0.1% FCS and 0.1% sodium-azide). Aliquots of the anti-CD3 mAbs serially diluted in FCM buffer, were added to 96 well V-bottom tissue culture plates along with 250,000 U937 cells/well. After incubating the cells for 15 mins. at 0°C , $0.3\mu\text{g}$ of anti-CD5 was added. Displacement of Fc-mediated anti-CD3 binding was allowed to occur for 90 minutes at 0°C , after which cells were harvested and washed in FCM buffer. Fluorescence of 10,000 cells stained with the PE-anti-CD5 Ab was determined using a FACScan flow cytometer. The data was plotted in a format using Consort 30 software as described below.

For competitive inhibition assay for FcR binding with ^{125}I -human IgG, U937 cells were washed and re-suspended at a concentration of 1.4×10^6 cells/mL in the assay medium (0.2% BSA in PBS). Aliquots of 1×10^6 cells per tube were incubated for 1h at 37°C with ^{125}I -labeled human IgG at a final concentration of 1×10^{-9} M. Murine or "humanized" OKT3 was added at final concentrations ranging from $0.023 \mu\text{g/ml}$ to $150 \mu\text{g/mL}$, with the total volume equaling $21 \mu\text{L/tube}$. Following the incubation, the mixture was layered over 10% sucrose. Upon centrifugation at 11000 g for 5 mins, the pelleted cells (bound ^{125}I -hulgG) separated from the medium containing free ^{125}I -hulgG. The tubes were then frozen in dry ice and the bottom of the tube containing the pelleted cells was removed for analysis of the bound ^{125}I -hulgG.

The maximum binding of ^{125}I -hulgG was determined in the absence of the inhibitor. The results are expressed as a percentage of the ^{125}I -hulgG bound in the presence of the inhibitor relative to the maximum

-48-

binding. Non-specific binding is seen as the percentage bound in the presence of excess inhibitor (150 μ g/ml murine OKT3). All controls and samples were assayed in triplicate tubes.

- 5 The N-terminal of the CH₂ domain of the mutated constructs is summarized in FIG. 7.

As shown in FIG. 6A and 6B, murine OKT3 IgG2a had, as expected, the highest affinity of all the anti-CD3 mAbs tested for FcR on U937 cells. As previously shown for human IgG4 mAbs, the gOKT3-5 required a 10-fold higher concentration to achieve the same inhibition. The Leu-234 mAb, that was expected to enhance FcR binding, has consistently proven to compete more efficiency for FcR binding than the gOKT3-5 mAb. In contrast, the Glu-235 mAb, bearing the FcR binding region similar to murine IgG2b, bound poorly to U937 cells, requiring a 10-fold higher concentration than the gOKT3-5 and approximately a 100-fold greater concentration than the murine OKT3 to achieve the same percent inhibition. These results indicated that, as anticipated from their respective amino acid sequence in the FcR binding domain, the rank order of binding of the mAbs to U937 cells was murine OKT3 > Leu-324 > gOKT3-5 > Glu-235 mAb.

EXAMPLE 8 Proliferation Assays.

The Glu-235 mAb was tested for its ability to induce T cell proliferation. Human peripheral blood mononuclear cells (PBMC) were obtained from normal volunteers by Ficoll-hypaque density gradient centrifugation of EDTA-anticoagulated whole blood. EBV-transformed lymphoblastoid cell lines (LCL) and human histiocytoma-derived U937 cell-line were maintained in continuous culture in complete media (DMEM supplemented with 2mM L-glutamine), 2 mM non-essential amino acids, 100 U/mL penicillin-streptomycin (Gibco), 5x10⁻⁵ M 2-mercapto-ethanol

-49-

(Gibco) and 25 μ M HEPES (Gibco) with 10% fetal calf serum (FCS, Gibco).

PBMC preparations were re-suspended in complete DMEM with 1% FCS and aliquotted to 96-well round bottom tissue culture plates (Costar) at 1×10^5 cells/well. The different Abs were added to the wells by serial log dilutions in culture media. After 72 hours of culture at 37°C in a 5% CO₂ incubator, 1 μ Ci of ³H-thymidine was added to each well and followed by an additional 24 hour incubation. Cells were harvested on a semi-automatic cell harvester and ³H-thymidine incorporation was measured in a liquid scintillation counter. All data were expressed as mean CPM of triplicate determinations.

Stimulation of PBMC with the wild-type gOKT3-5 mAb resulted in cell proliferation comparable to that observed with PBMC stimulated with murine OKT3, as shown in FIG. 8. In contrast, no proliferation was induced by the Glu-235 mAb using PBMC from 3 different donors at mAb concentrations up to 10 μ g/ml, suggesting that the alteration of the FcR binding region of this mAb had impaired its mitogenic properties.

20

EXAMPLE 9 Activation of T cells
by CDR-grafted mutant mAbs.

In order to further analyze early T cell activation events, human peripheral blood mononuclear cells (PBMC), cultured with various anti-CD3 mAbs, were assessed for cell surface expression of Leu 23 and IL-2 receptor at 12 and 36 hours incubation, respectively.

25

For studies involving T cell expression of activation markers, 2×10^6 PBMC were cultured for either 12 hours (Leu 23 expression) or 36 hours (IL-2 receptor expression) in 24 well tissue culture plates in the presence of varying concentrations of the mAbs.

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-50-

No significant differences were reproducibly observed between murine OKT3 and gOKT3-5 mAb with respect to expression of these cell surface markers (see FIG. 9). In contrast, activation by the Glu-235 mAb resulted in lower levels of expression of both markers. In fact, the highest concentration of the Ab used (10 μ g/mL) achieved less than 40% of the maximal activation obtained with standard OKT3. No differences in the expression of these markers were observed between CD4⁺ and CD8⁺ cells (data not shown).

10 **EXAMPLE 10** IFN- γ , GM-CSF and TNF- α production
 induced by "humanized" OKT3 mAbs.

The acute toxicity observed in transplant recipients after the first administration of OKT3 has been attributed to the systematic release of lymphokines triggered by the mAb. Therefore, the *in vitro* production of GM-CSF, TNF- α and IFN- γ induced by the "humanized" anti-CD3 mAbs was measured. For studies involving lymphokine production, 2 x 10⁶ PBMC were cultured in 24-well plates for either 24 hours (TNF- α) or 72 hours (GM-CSF and IFN- γ). Tissue culture supernatants were collected at the completion of the respective incubation periods and stored at -20°C. Lymphokine levels were measured via sandwich ELISA techniques using commercially available kits.

Similar amounts of cytokines were produced after culture of PBMC with OKT3 and gOKT3-5 mAb. In contrast, the highest concentration of the Glu-235 mAb induced small quantities of TNF- α (see FIG. 10) and GM-CSF (data not shown), and no IFN- γ (data not shown).

25 **EXAMPLE 11** Induction of modulation and coating of the TCR
 complex by molecularly engineered OKT3 mAbs.

30 The immunosuppressive properties of the different mAbs was compared *in vitro*. First, the mAbs were examined for their capacity to modulate and/or coat the TCR complex. Human peripheral blood mononuclear cells (PBMC) were incubated at 1x10⁶ cells/mL for 12 hours

-51-

in 24 well plates with known concentrations of anti-CD3 mAb. PBMC from each group were harvested and stained with either OKT3-FITC or OKT3D-FITC. The fluorescein-stained cells were counterstained with anti-CD5-PE to identify T lymphocytes and analyzed by flow cytometry (FCM). OKT3D-FITC was selected because of its binding to an epitope distinct from the one binding OKT3 mAb. Thus, this Ab provided a direct measurement of unmodulated surface CD3.

Formulae for calculating CD3 coating and modulation were:

$$\% \text{ CD3 Mod.} = \frac{\text{Control Cells } MC_{\text{OKT3D-FITC}} - \text{Ab-treated cells } MC_{\text{OKT3D-FITC}}}{\text{Control Cells } MC_{\text{OKT3D-FITC}}} \times 100$$

$$\% \text{ CD3 Coated} = \frac{\text{Ab-treated Cells } MK_{\text{OKT3D-FITC}}}{\text{Control Cells } MC_{\text{OKT3D-FITC}}} - \frac{\text{Ab-treated Cells } MC_{\text{OKT3-FITC}}}{\text{Control Cells } MC_{\text{OKT3-FITC}}} \times 100$$

$$\% \text{ CD3 Uncoated} + \text{Unmodulated} = 100 (\% \text{ CD3 Coated} + \% \text{ CD3 Modulation})$$

Where MC represents the mean channel along the x-axis.

10

As shown in FIG. 11, the combined modulation and coating of the TCR complex achieved by the gOKT3-5 and murine OKT3 were very similar, with half-maximal TCR blocking achieved at approximately 1 ng/ml. However, the half-maximum modulation plus coating observed with the Glu-235 mAb required a 100-fold greater concentrations of mAb

15

-52-

(1 μ g/mL) than of murine OKT3. The major difference between the Glu-235 mAb and the other Abs was due to a change in kinetics since, by 48 hours, the mAb coated and modulated the TCR complex similarly to OKT3 (data not shown). Thus, the achievement by Glu-235 mAb of
5 internalization of the TCR, which may depend on multivalent cross-linking, was delayed as compared with the other anti-CD3 mAbs.

EXAMPLE 12 Inhibition of CTL activity
 by CDR-grafted mutant mAbs.

10 The ability of the Abs to suppress cytotoxicity of alloreactive T cells was compared. HLA-A2-specific CTL were generated from a normal HLA-A1 donor. Cytolytic activity was assessed on FcR negative-EBV-transformed HLA-A2 target cells. CTL were generated by a bulk allogeneic MLC technique. Normal human donors were phenotyped for
15 HLA-A expression. Responder and stimulator combinations were selected specifically to generate HLA-A2-specific CTL effectors. Responder and stimulator PBMC were prepared by Ficoll-hypaque density gradient centrifugation as described above and re-suspended in RPMI 1640 with
20 2mM L-glutamine, 100 U/mL penicillin-streptomycin, 25 μ M HEPES and 15% decompemented normal human serum. Stimulator PBMC (1×10^7 /mL) were irradiated (3000 rad) and cultured with responder PBMC (1×10^7 /10mL) in upright 25 cm tissue culture flasks. After 7 days of culture, freshly irradiated stimulator PBMC (4×10^6 /10mL) were added to
25 4×10^6 /10mL of the initial cultured cells and incubated for an additional five days. Cells were then harvested and assayed for CTL activity by ^{51}Cr release.

 HLA-A2-specific CTL effectors were generated as described above, harvested and aliquotted to a 96 well U-bottom tissue culture plate at
30 four different effector/target ratios. Effectors were pre-incubated with serial dilutions of each anti-CD3 mAb for 30 minutes. Following incubation with mAbs, ^{51}Cr -labeled Fc receptor negative-target cells [HLA-

-53-

A2 expressing LCL line (Z2B) or HLA-A1 expressing LCL line (G12B) used as a non-specific target] were added. Spontaneous lysis was measured by incubation of targets alone in media and maximal lysis was achieved by addition of 0.05 N HCL. Effectors and targets were co-cultured; 5 supernatant aliquots were harvested and radioactivity was measured in a gamma-counter.

T cell cytotoxicity was specific as demonstrated by the absence of lysis of a syngeneic HLA-A1 EBV-transformed cell-line (data not shown). 10 Inhibition of lysis by anti-CD3 mAbs previously has been attributed to the inability of the T cells to recognize their targets, due to TCR blockade by the mAb. In the present study, murine OKT3, gOKT3-5 mAb and Glu-235 exhibited a comparable inhibitory effect on the cytolytic activity of the alloreactive T cells. These results suggest that the ability of the different 15 mAbs to coat the TCR within the 30 min incubation time was similar (see FIG. 12). In contrast, the gOKT3-6 mAb, a "humanized" OKT3 that has a significantly reduced binding activity for the CD3 antigen, did not inhibit CTL activity. These results suggest that modified affinities for FcRs do not alter the immunosuppressive property of the anti-CD3 mAbs, *in vitro*.

20

EXAMPLE 13 CD4 modulation studies.

PBMCs isolated from Ficoll-Hypaque density gradient centrifugation were incubated at 1×10^6 cell/mL with known concentrations of OKT3 25 antibodies at 37° C for 24 hours. The cells were harvested and stained with FITC-OKT4. The cells were counterstained with PE-labelled anti-CD5 (PE-Leu1, Becton Dickinson Immunocytometry Systems, San Jose, CA) to distinguish T lymphocytes from other PBMCs, and analyzed by FACScan. Data from the resulting studies are reported in FIG. 1 (Transy, 1989).

30

Results were calculated using the following formulae:

$$\% \text{ Specific lysis} = \frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Maximal CPM} - \text{Spontaneous CPM}}$$

$$\% \text{ Maximal specific lysis} = \frac{\% \text{ Specific lysis}_{[\text{mAb}]}}{\% \text{ Specific lysis}_{\text{Control}}}$$

Where $\% \text{ Specific lysis}_{[\text{mAb}]}$ represents the CPM obtained at a given mAb concentration for a E:T ratio of 25:1 and $\% \text{ Specific lysis}_{\text{Control}}$ represents the CPM obtained in the absence of mAb at the same E:T ratio. Results were expressed as the mean of triplicates.

%CD4 modulation was calculated as follows:

$$5 \quad \frac{\text{Control MCN}_{\text{FITC-OKT4}} - \text{Ab treated MCN}_{\text{FITC-OKT4}}}{\text{Control MCN}_{\text{FITC-OKT4}}} \times 100$$

The data in the left plot of FIG. 13 reveal that the humanized antibodies studied induce the modulation of CD4 in a dose-dependent manner. In contrast is the data for mOKT3 (solid circles), the antibody from which the humanized and mutated antibodies were constructed, had no effect on CD4, as indicated by a straight line plot between antibody concentrations of from 0.01 to 0.10 $\mu\text{g/mL}$. The same can be said for the mOKT3D IgG2b antibody (solid triangles) which has also been neither humanized nor mutated.

-55-

The right plot indicates that, as expected, there is no modulation of CD8 for any of the antibodies studied.

EXAMPLE 14 ELISA and RES-KW3 studies of CD4 binding.

5 RES-KW3 cells were washed with PBS + 0.2% BSA + 0.1% sodium azide (staining buffer), and first incubated with various concentrations of OKT3 antibodies for 1 hour on ice. The cells were washed three times with cold staining buffer, and FITC-labelled goat anti-human or goat anti-mouse antibodies were added (Caltac Lab. So. San Francisco, CA). The
10 cells were incubated on ice for another hour before being washed and subject to FCM.

FCM was performed using a FACScan (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) flow cytometer
15 interfaced to a Hewlett-Packard 340 computer, data analyzed using Lysis II software (Becton Dickinson). Fluorescence data were collected using logarithmic amplification on 10,000 viable cells as determined by forward and right angle light scatter intensity. One-color fluorescence data were displayed in histogram mode with fluorescence intensity on the x axis and
20 relative cell number on the y axis.

HIVgp120/CD4 receptor EIA coated microplates from DuPont were used in the CD4 binding assay. 100 μ L/well of CDR-grafted OKT4A1gG1 at various concentrations (1:2 dilution at starting concentration of 50
25 ng/mL) was added into the wells duplicate for the construction of standard curve. 100 μ L/well of OKT3 antibody samples at various dilutions were then added. The diluent is PBS + 10% calf serum + 0.05% Tween-20. The plates were incubated at room temperature for 2 hours.

30 The plates were washed with PBS + 0.05% Tween-20 six times before 100 μ L/well of 1:15000 diluted HRPO-conjugated goat anti-human x(f + B) antibodies in diluent was added. The plates were incubated at

-56-

room temperature for another 2 hours. The plates were washed six times again, and 100 μ L/well of the OPD/hydrogen peroxide solution (five 2-mg OPD tablets were added in 13 mL of Mili-Q water; after they were dissolved, 5 μ L of 30% hydrogen peroxide were then added) was added into each well. The plates were incubated at room temperature in the dark for 30 minutes, and 50 μ L/well of 2.5N HCl was added to stop the reaction. The plates were then read at 490 nm.

The resulting data are reported in FIG. 14 and 15. These data indicate that the humanized OKT3 binds to CD4, either immobilized to ELISA plates or bound to the surface of RES-KW3 cells. It will be appreciated by one skilled in the art that data such as that indicated in FIG. 6 for 209IgG1A/A-1 (open circles) are unexpected, and suggest that divalent binding (binding to both CD3 and CD4, for example), is needed for stable attachment of this antibody to the plate.

EXAMPLE 15: Generation of a Non-Activating Anti-CD3 mAb Based on gOKT3-7.

To generate an anti-human CD3 mAb with an improved therapeutic index, the inventors have developed a panel of "humanized" anti-CD3 mAbs derived from OKT3, by molecularly transferring the complementary determining regions (CDRs) of OKT3 onto human IgG1 and IgG4 molecules (Woodle *et al.*, 1992; Adair *et al.*, submitted for publication). In addition, the inventors examined whether immunosuppression can be achieved by anti-CD3 mAbs in the absence of the initial step of cellular activation. The "humanized" mAb, formally named gOKT3-7(τ_1), abbreviated 209-IgG1, that has a high affinity for human Fc γ Rs was shown, in vitro, to have similar activating properties to OKT3 (Alegre, 1992; Xu *et al.*, manuscript in preparation) and would therefore be expected to induce in patients the acute toxicity associated with lymphokine release by activated T cells and Fc γ R-bearing cells. A second mAb, formally named gOKT3-7(τ_4 -a/a); abbreviated Ala-Ala-IgG4, was

-57-

developed with 2 amino acid substitutions in the CH₂ portion (from a phenylalanine-leucine to an alanine-alanine at positions 234-235) of the "humanized" gOKT3-7(r4) (209-IgG4) mAb. These mutations significantly reduced binding of the mAb to human and murine FcγRI and

5 II and led to markedly reduced activating characteristics *in vitro* (Alegre, 1992; Xu *et al.*, manuscript in preparation). Importantly, this variant mAb retained the capacity to induce TCR modulation and to prevent cytolysis *in vitro* (Xu *et al.*, manuscript in preparation), and thus represents a potential new immunosuppressive therapeutic agent.

10

Severe combined immunodeficient (SCID) mice carry an autosomal recessive, spontaneously arising mutation that results in the inability to successfully rearrange immunoglobulin and TCRs. These animals are therefore devoid of T and B lymphocytes (McCune, *Annu. Rev. Immun.*, 15 1991; McCune, *Curr. Opin. Immun.*, 1991; Bosma, 1983; Bosma, 1991). The inventors have recently developed a model in which lightly irradiated SCID mice are injected with human splenocytes from cadaveric organ donors (Alegre *et al.*, manuscript submitted). These hu-SPL-SCID mice maintain functional human T cells capable of responding to mitogens and

20 alloantigens *in vitro*, and of acutely rejecting human foreskin allografts *in vivo*. In the present study, the inventors have utilized hu-SPL-SCID mice to assess the immunosuppressive properties of the non-activating "humanized" anti- CD3 mAbs *in vivo*.

25

MATERIALS AND METHODS

Abbreviations.

	Ala-Ala-IgG4	gOKT3-7(r ₄ a/a)
	FCM	flow cytometry
	GVHD	graft-versus-host disease
30	IP	intraperitoneal
	PE	Phytoerythrin
	209IgG1	gOKT3-7(r1)

-58-

209IgG4
SCID

gOKT3-7(r4)
severe combined immunodeficient

Mice. Homozygous C.B-17 scid/scid (SCID) H-2^d founder mice
5 were obtained from Dr. M. Bosma (Fox Chase, Phila, PA) and were
subsequently bred in the specific pathogen-free animal barrier facility at
the University of Chicago.

Antibodies. 145-2C11, a hamster anti-mouse CD3 mAb, was
10 purified from hybridoma supernatant using a protein A column (Sigma,
Saint Louis, MO), as previously described (Leo, 1987). OKT3, 209-IgG1
and Ala-Ala-IgG4 were generated as described below. Phycoerythrin
(PE)-coupled anti-human CD4 and CD8, as markers of T cells, were
obtained from Coulter Immunology (Hialeah, FL). The fluorescein
15 isothiocyanate (FITC)-coupled anti-CD69, an early marker of T cell
activation, was purchased from Becton Dickinson (San Jose, Ca). All
anti-human Abs were tested to exclude cross-reactivity on murine cells.

Generation and function of "humanized" anti-CD3 mAbs.

20 Permanent myeloma transfectants of the murine and human-OKT3 mAbs
genes were developed as previously described (Xu *et al.*, manuscript in
preparation). Mutation of the phenylalanine-leucine sequence at position
234-235 into alanine-alanine to decrease the affinity of the mAb for
human and murine FcγRI and II were performed as previously described
25 (Alegre, 1992; Xu *et al.*, manuscript in preparation). ELISAs using a
combination of goat anti-human Fc and kappa Abs were performed to
determine the yield of assembled "humanized" antibody in COS cell
supernatants or permanently transfected myeloma cell-lines (Woodle,
1992).

30

For T cell proliferation assays, PBMCs, in complete medium
(RPMI-1640 plus 10% FCS), were incubated at 1×10^6 cells/ml (final

-59-

volume = 200 μ l) with serial log dilutions of each antibody in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) for three days at 37 °C. All mAbs samples were airfuged at >30 psi for 20 minutes prior to the assay to remove preformed aggregates (Beckman, Carlsbad, CA).

- 5 3 H-Thymidine (NEN-DuPont, Wilmington, DE) was added at 1 μ Ci/well and the plates were incubated for additional 4 hours before harvesting. The cells were harvested in an automatic 96-well cell harvester (Tomtec, Orange, CT) and 3 H-thymidine incorporation was measured with a Betaplate Liquid Scintillation Counter (Pharmacia).

10

- Construction and treatment of hu-SPL-SCID mice. Fresh human spleens were obtained from cadaveric organ donors, under a protocol approved by the University of Chicago Institutional Review Board. A single cell suspension was prepared as previously described (Alegre *et al.*,
15 manuscript submitted). Briefly, 4 to 6 week-old SCID mice were γ -irradiated (200 rad), prior to the intraperitoneal (ip) injection of 10^8 cells/mouse. The percentage of human cells in the peripheral blood was determined by flow cytometry (FCM). First, the peripheral blood mononuclear cells (PBMCs) were incubated (15 minutes) with unlabelled
20 murine IgG antibodies to block subsequent Fc γ R binding. Next, the cells were stained with PE-coupled anti-murine class I (PharMingen, San Diego, Ca) and counterstained with FITC-coupled anti-human CD45 mAb (Coulter Immunology, Hialeah, FL) to identify the population of human cells. The proportion of human cells is expressed as a percentage of the total
25 number of cells. The animals bearing between 5 and 20% human cells in the PBMCs were selected for further experiments. Mice, matched for their level of engraftment of human cells in the peripheral blood, received either PBS (1 ml), 145-2C11, OKT3, 209-IgG1 or Ala-Ala-IgG4 (100 μ g resuspended in 1 ml of PBS, unless stated otherwise in the text),
30 intraperitoneally (ip) 11 days to 3 weeks after the injection of the human splenocytes.

-60-

Detection of circulating anti-CD3 mAbs. SCID and hu-SPL-SCID mice were bled by retroorbital venous puncture 24h, 48h and 1 week after the injection of the mAbs (100 μ g ip). The serum titers of the anti-CD3 mAbs were determined by FCM analysis using human PBMNs obtained from EDTA-anticoagulated whole blood of normal volunteers and isolated by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient centrifugation. Six concentrations of purified OKT3, 209-IgG1 and Ala-Ala-IgG4 in 3-fold dilutions were used to generate standard curves. Human PBMCs were incubated with 3 serial dilutions of each serum (1:10, 1:30 and 1:90), and then stained with FITC-coupled goat anti-mouse Ig (Boehringer-Mannheim, Indianapolis, IN) for detection of OKT3, and with goat anti-human Ig (Caltag Laboratories, San Francisco, CA) for detection of the humanized antibodies. Serum levels were extrapolated from the mean fluorescence of anti-CD3 stained cells, as compared with a corresponding concentration of the purified anti-CD3 mAbs on the standard curves.

Detection of circulating IL-2. Sera obtained from SCID and hu-SPL-SCID mice 2h after anti-CD3 or control treatment were analyzed for the presence of IL-2 was analyzed using a colorimetric assay that utilized the IL-2/IL-4-dependent cell line, CTLL-4, as previously described (Mosmann, 1983). CTLL-4 cells proliferated similarly to recombinant murine and human IL-2, and responded to murine but not human IL-4. To exclude participation of murine cytokines in the proliferation observed, an anti-murine IL-4 mAb, [11B11 (Ohara, 1985)], and an anti-murine IL-2 mAb, [S4B6, (Cherwinski, 1987)], were added to selected wells at concentrations found to block proliferation of CTLL-4 cells to murine IL-4 and IL-2, respectively, but not to human IL-2.

Skin grafting. Neonatal human foreskin was grafted on SCID and hu-SPL-SCID mice 11 days after the inoculation of human splenocytes. Mice were anesthetized with 60 μ g/ml of chlorohydrate (120 μ l delivered

-61-

ip) (Sigma, St. Louis, MO) and intermittent inhalation of hydroxyflurane (Metophane, Pitman-Moore, Mundelein, IL). Skin grafts were positioned on the dorsal thorax of the mice. Each foreskin was used to graft 4 animals, each from a different group (SCID, PBS-treated, 145-2C11-treated and anti-CD3-treated hu-SPL-SCID mice). Mice received OKT3, 209-IgG1, Ala-Ala-IgG4 or 145-2C11 (50 μ g/day for 5 days, followed by 10 μ g/day for 10 days) diluted in 1 ml of PBS, or 1 ml of PBS alone. The grafts were unwrapped at 7 days and the status of the graft was scored blindly and independently by 2 investigators daily for the first 30 days, and once a week afterwards. The scores ranged from 0 to 4: grade 0 represented skin grafts intact and soft; grade 1, skin grafts with a modified pigmentation in a small area; grade 2, soft skin grafts with larger areas of depigmentation; grade 3, those hardened or slightly scabbed; grade 4, shrinking or scabbing skin grafts. Rejection was recorded when scores were grade 3 or greater.

RESULTS

Characteristics of the "humanized" mAbs. OKT3 and the "humanized" mAbs were shown in companion studies to have similar avidities for the human CD3 complex, as determined by flow cytometry (FCM) in a competitive binding assay using FITC-coupled OKT3 (Alegre, 1992). In a competitive inhibition assay for FcR binding using 125 I-human IgG and the human monocytic cell-line U937, OKT3, 209-IgG4 and 209-IgG1 were found to have similar affinities for human Fc γ Rs, whereas the binding of the Ala-Ala-IgG4 and Ala-Ala-IgG1 mAbs to human Fc γ RI or Fc γ RII were greatly reduced (Xu *et al.*, manuscript in preparation). Finally, the "humanized" mAbs were tested for their ability to induce T cell proliferation. Stimulation of PBMCs with the 209-IgG4 or 209-IgG1 mAbs resulted in cell proliferation comparable to that observed with PBMCs stimulated with murine OKT3 (Figure 16). In contrast, no significant proliferation was induced by the Ala-Ala-IgG4 mAb at

-62-

concentrations up to 100 ng/ml. In fact, the proliferation observed at the highest concentrations may be due to aggregation of the mAb. These results suggest that the alteration of the Fc γ R-binding region of this mAb had impaired its mitogenic properties.

5

Determination of the circulating levels of anti-CD3 mAbs. Ten days to three weeks after injection of 108 human splenic cells in the peritoneal cavity, SCID mice were tested for the percentage of human cells engrafting their peripheral blood. As previously described, graft versus
10 host disease (GVHD) was apparent in mice bearing more than 25 to 30% human cells (Alegre *et al.*, manuscript submitted). Therefore, in order to minimize the level of human T cell activation prior to anti-CD3 treatment, animals with 5% to 20% circulating human CD45⁺ cells were selected for subsequent experiments. Mice matched for their level of engraftment
15 with human cells were assigned to different groups for treatment with OKT3, 209-IgG1, Ala-Ala-IgG4 or PBS. As shown in Figure 17, significant serum levels of all of the anti-CD3 mAbs (between 8 and 13 μ g/ml) were measured 24h after the injections. No anti-CD3 mAb was detected in SCID or hu-SPL-SCID mice treated with PBS (data not
20 shown). The persistence of the mAbs was relatively short, inasmuch as levels decreased dramatically by 48h. These data are consistent with results reported previously of a short half-life of immunoglobulins in other hu-SPL-SCID experimental models (Duchosal, 1992). They also are reminiscent of the time course for clearance of circulating OKT3 following
25 its injection into humans (Thistlethwaite, 1988).

Depletion of T cells following administration of anti-CD3 mAbs.

The injection of OKT3 and 209-IgG1 into hu-SPL-SCID mice induced a rapid and substantial depletion of circulating human CD45⁺ cells, that
30 was almost maximal when first measured, 3h after the injection (data not shown). These data are consistent with the clearance of T cells from the peripheral blood seen in humans following the injection of OKT3.

-63-

Interestingly, the depletion observed in the peripheral blood after administration of Ala-Ala-IgG4 in hu-SPL-SCID mice was consistently less striking than after the injection of the activating anti-CD3 mAbs, suggesting that binding of the anti-CD3 mAbs to Fc γ Rs might play a role in the reduction of the number of circulating T cells. The clearance of human cells from the spleen and peritoneal cavity was not complete after a single injection of any of the anti-CD3 mAbs, activating or non-activating. In addition, the kinetics of depletion in the spleen were slower than in the peripheral blood, with maximal loss of 60% of the human cells not achieved until 48h (data not shown). In contrast, a protocol analogous to that employed clinically in human transplant recipients, consisting of 14 consecutive days of i.p. administration of the anti-CD3 mAbs (10 μ g), resulted in a complete depletion of CD3⁺ T cells in the peripheral blood, the spleen and the peritoneal cavity even after Ala-Ala-IgG4 (data not shown). This absence of CD3⁺ cells was not due to modulation and/or coating of the TCR complex by mAbs, inasmuch as staining with PE-coupled anti-CD4 or anti-CD8 mAbs did not reveal any remaining human T cells. Furthermore, hu-SPL-SCID splenocytes harvested 3 days after the completion of this protocol were unable to proliferate to immobilized OKT3, *in vitro* (data not shown). It is interesting to note that the ability of OKT3 to deplete T cells from human lymphoid compartments such as spleen or lymph nodes is unknown. However, studies using the anti- mouse CD3 mAb, 145-2C11, have shown that T cells are also depleted from the peripheral lymphoid organs of the immunocompetent mice.

Induction of surface markers of activation on T cells after administration of anti-CD3 mAbs. An early event following injection of OKT3 into transplant recipients is the activation of CD3⁺ T cells due to the cross-linking of the TCR by Fc γ R⁺ cells (Abramowicz, 1989; Chatenoud, 1989; Ceuppens, 1985). T cell activation in patients results in increased surface expression of markers such as CD69, CD25 and

-64-

HLA-DR. As previously described, a significant percentage of hu-SPL-SCID T cells express CD25 and HLA-DR, as a result of GVHD (Alegre *et al.*, manuscript submitted). In contrast, levels of CD69, which is an earlier and more transient marker of activation, are comparable to those found on T cells from humans. A significant increase in the expression of CD69⁺ on both CD4⁺ and CD8⁺ splenocytes was observed 24h after the injection of OKT3 and 209-IgG1 into hu-SPL-SCID mice, but not after the administration of Ala-Ala-IgG4 or PBS (Figure 18), suggesting that the Ala-Ala-IgG4 mAb induced less T cell activation than the FcγR-binding anti-CD3 mAbs.

Production of IL-2 after anti-CD3 therapy. The administration of OKT3 to patients has been shown to induce the rapid systemic release of cytokines such as TNF- α , IL-2, IL-6 and IFN- γ , peaking 2 to 6h after the injection (Abramowicz, 1989; Chatenoud, 1989). This cytokine production results in the acute toxicity associated with anti-CD3 therapy in transplant recipients. In the present study, a bioassay was used to measure the serum level of human IL-2 2h after treatment of hu-SPL-SCID mice with PBS, OKT3, 209-IgG1, Ala-Ala-IgG4 or 145-2C11, a hamster anti-murine CD3 mAb. As shown in Figure 19, only the injection of OKT3 and 209-IgG1 induced the release of detectable human IL-2 in hu-SPL-SCID mice. The levels detected were low because of the relatively small percentage of engrafted human cells, but readily detectable in the experiments performed. The lymphokine production from individual animals varied as a consequence of the different percentage of human cells engrafting each animal. No human or murine IL-2 was detected after injection of 145-2C11, confirming the absence of endogenous murine T cells in these mice. The administration of Ala-Ala-IgG4 did not induce IL-2 production, consistent with the reduced ability of this mAb to fully activate human T cells. To verify the human origin of the cytokines detected, polymerase chain reaction assays were performed on spleens of SCID and hu-SPL-SCID mice 6h after treatment,

-65-

using primers that did not cross-react with murine cytokines. In addition to IL-2, IFN- γ mRNA was found to be up-regulated after injection of the OKT3 and 209-IgG1 mAbs, but not the Ala-Ala IgG4 mAb (data not shown). Together, these results demonstrate that the Ala-Ala-IgG4 mAb
5 has reduced activating properties as compared with OKT3 and 209-IgG1.

Prolongation of skin graft survival by the administration of anti-CD3 mAbs. The immunosuppressive properties of the different mAbs was next examined. Previous studies have shown that the 209-IgG1 and the
10 Ala-Ala-IgG4 mAbs were both effective at modulating TCR and suppressing cytotoxic T cell responses *in vitro* (Alegre, 1992; Xu *et al.*, manuscript in preparation). Initial studies *in vivo* suggested a similar rapid immunosuppressive effect induced by both "humanized" mAbs, as TCR
15 was significantly modulated from the cell surface 24h following injection of either mAb (data not shown). However, in order to directly explore the immunosuppressive efficacy of these mAbs, the inventors performed skin graft experiments. Previous studies from the inventors' laboratory have shown that hu-SPL-SCID mice are capable of rejecting human foreskin allografts and that human T cells participate in this process (Alegre *et al.*,
20 manuscript submitted). SCID and hu-SPL-SCID mice were grafted with human foreskin obtained from circumcisions and assumed to be allogeneic with respect to the human cells used for the adoptive transfer. Hu-SPL-SCID mice matched for their level of human CD45 expression in the peripheral blood received either PBS or daily doses of OKT3,
25 209-IgG1, Ala-Ala-IgG4, or 145-2C11 for 15 consecutive days, beginning on the day of the skin graft. As shown in Figure 20, animals that received PBS or 145-2C11 rejected their grafts with a 50% mean survival time of 13 days, consistent with the inventors previous results. In contrast, all of the OKT3- treated animals and all but 1 of the
30 209-IgG1- and Ala-Ala-IgG4-treated mice maintained their skin grafts for greater than 80 days. Mice were sacrificed at 80 days, and 2 animals per group were analyzed for the percent of human cells in the different

-66-

cellular compartments. None of the anti-human CD3-treated mice reexpressed human CD3⁺ cells in the peripheral blood, the spleen or the peritoneal cavity, as determined by FCM. In contrast, the PBS-treated animals retained a significant percentage of human CD45⁺ and CD3⁺ cells in the different compartments although the absolute numbers were reduced over time, as compared with the initial engraftment (data not shown). Three additional skin graft experiments have been performed with 5-7 animals per group. In these experiments, 66-80% of the animals treated with OKT3, 209-IgG1 and Ala-Ala-IgG4 maintained their grafts for as long as the animals were examined. In two of the three experiments, a higher percentage of mice treated with the Ala-Ala-IgG4 maintained their skin grafts permanently. No statistical difference was found between these 3 groups.

DISCUSSION

These studies suggest that a "humanized" mAb derived from OKT3 and bearing mutations of 2 amino acids in the Fc portion to impede its binding to Fc γ R_s does not induce human T cell activation *in vivo* in a preclinical model, but retains the immunosuppressive properties of the native mAb.

OKT3 has been shown to mediate T cell activation by cross-linking T lymphocytes and Fc γ R⁺ cells (Palacios, 1985; Ceuppens, 1985; Kan, 1985). Because hu-SPL-SCID mice are chimeric animals comprising both murine and human FcR⁺ cells, it was important to use mAbs that would have similar avidities for human and murine FcR_s. Thus, OKT3, a murine IgG2a, and the human 209-IgG1 mAb have a high affinity for FcR_s of both species (Xu *et al.*, manuscript in preparation). In contrast, the human Ala-Ala-IgG4 bears mutations dramatically reducing its binding to murine and human FcR_s. The efficacy of engraftment of the different cellular compartments with human B cells, monocytes/macrophages and

-67-

NK cells, as providers of human Fc γ R, is relatively low in this hu-SPL-SCID model [10% in the peritoneal cavity and the peripheral blood and 20% in the spleen (Alegre *et al.*, manuscript submitted)], when compared to the proportion of human T lymphocytes observed. On the other hand, murine monocytes/macrophages and NK cells are functionally normal in SCID mice and express normal levels of murine Fc γ R (Bosma, 1991; Kumar, 1989). The type of accessory cell responsible for the cross-linking mediated by OKT3 and 209-IgG1 in this chimeric system, whether murine or human, was adequate to trigger cellular activation analogous to that observed in patients after the injection of OKT3. Indeed, OKT3 and 209-IgG1-triggered activation of the human T lymphocytes was evident in the treated mice, as determined by the production of human IL-2 and the accumulation of human IFN- γ mRNA, as well as by the increased expression of the surface marker of activation, CD69, on T cells. In contrast, the inability of Ala-Ala-IgG4 to interact with Fc γ Rs rendered this mAb incapable of fully triggering T cell activation.

The activation of T lymphocytes and Fc γ R⁺ cells in patients treated with OKT3 is associated with adverse reactions such as fever, chills, headaches, acute tubular necrosis, diarrhea, acute respiratory distress syndrome etc. (Abramowicz, 1989; Chatenoud, 1989; Toussaint, 1989; Thistlethwaite, 1988; Goldman, 1990). Similarly, immunocompetent mice injected with 145-2C11 develop hypothermia, hypoglycemia, lethargy, liver steatosis and acute tubular necrosis (Alegre, *Eur. J. Immun.*, 1990; Alegre, *Transplantation*, 1991; Ferau, 1990). Hu-SPL-SCID mice did not exhibit detectable symptoms after OKT3 or 209-IgG1 therapy if the percentage of human cell engraftment was moderate. However, when animals with more than 30% human cells in their PBMCs were injected with OKT3 or 209-IgG1, they became extremely lethargic and an increased percentage of animal deaths was observed. As shown previously, animals engrafted with a high

-68-

percentage of human T cells often undergo a GVHD-like syndrome, that results in a number of pathological symptoms including pancreatitis, diffuse hemorrhagic necrosis and in many instances animal death. Interestingly, the administration of Ala-Ala-IgG4 to highly engrafted
5 animals seemed to reduce the symptoms of GVHD and perhaps even prevent some deaths. The number of animals examined was, however, too small to generate statistical differences.

The administration of all 3 anti-CD3 mAbs to hu-SPL-SCID mice,
10 whether activating or not, resulted in modulation of the CD3 molecules from the surface of T lymphocytes and subsequent T cell depletion (data not shown). Similarly, in transplanted patients treated with OKT3, rapid modulation of the TCR complex and T cell depletion from the peripheral circulation are presumably responsible for the immunosuppressive
15 properties of the drug (Chatenoud, 1982). Importantly, in this study, the administration of the Ala-Ala-IgG4 mAb resulted in dramatic prolongation of allograft survival similarly to the activating OKT3 and 209-IgG1 mAbs. These findings indicate that complete T cell activation due to T lymphocyte/FcR⁺ cell cross-linking may not be necessary for the
20 achievement of a potent anti-CD3-mediated immunosuppression.

In summary, the Ala-Ala-IgG4, a mAb bearing 2 amino acid mutations in the Fc portion of a "humanized" OKT3, may prove useful in clinical transplantation to induce immunosuppression while being less
25 immunogenic and induce less adverse reactions than OKT3. In addition, the use of a "humanized" mAb may lessen the generation of anti-xenotypic Abs that often arise after repeated administrations of OKT3 (Thistlethwaite, 1988). Finally, the non-activating Ala-Ala-IgG4 mAb might also widen the applications of anti-CD3 mAbs to patients suffering
30 from autoimmune diseases, in whom treatment with OKT3 was never realized because of the potential adverse reactions and the strong humoral responses induced by the mAb.

EXAMPLE 16: *In Vitro* Uses of Antibodies.

In addition to the above-described uses, the claimed antibodies will have a variety of *in vitro* uses. Some of these are described below,
5 others will be understood by those of skill in the art.

1. Immunoassays

The antibodies of the invention will find utility in immunoassays for the detection of CD3. Turning first to immunoassays, in their most
10 simple and direct sense, preferred immunoassays of the invention include the various types of enzyme linked immunosorbent assays (ELISAs) known to the art. However, it will be readily appreciated that the utility of antibodies is not limited to such assays, and that other useful
15 embodiments include RIAs and other non-enzyme linked antibody binding assays or procedures.

In the preferred ELISA assay, samples to be tested for CD3 are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After
20 washing to remove incompletely adsorbed material, one will desire to bind or coat a nonspecific protein such as bovine serum albumin (BSA), casein or solutions of milk powder onto the well that is known to be antigenically neutral with regard to the anti-CD3 antibody. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces
25 the background caused by nonspecific binding of the antibody onto the surface.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound
30 material, the immobilizing surface is contacted with the anti-CD3 antibody in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting with diluents such as BSA,

-70-

bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background. The layered antibody is then allowed to incubate for from 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C. Following incubation, the antibody-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the anti-CD3 antibody. Of course, in that the anti-CD3 will typically have a human IgG region, the second antibody will preferably be an antibody having specificity in general for human IgG. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

25

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H₂O₂, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

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-71-

2. Fluorescence Activated Cell Sorting (FACS)

Fluorescent activated cell sorting, flow cytometry or flow microfluorometry provides the means of scanning individual cells for the presence of an antigen. The method employs instrumentation that is capable of activating, and detecting the excitation emissions of labeled cells in a liquid medium.

FACS is unique in its ability to provide a rapid, reliable, quantitative, and multiparameter analysis on either living or fixed cells. The "humanized" anti-CD3 antibodies provide a useful tool for the analysis and quantitation of antigenic, biophysical, and biochemical characteristics of individual cells. When used with electrostatic deflection technology, the antibodies of the present invention can be used for the specific isolation of subpopulations of cells.

3. Immunohistochemistry

The antibodies of the present invention may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared from study by immunohistochemistry (IHC). For example, each tissue block consists of 50 mg of residual "pulverized" tumor. The method of preparing tissue blocks from these particulate specimens was developed and has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown, *et al.* (1990); Abbondanzo, *et al.* (1990); Allred *et al.* (1990)).

Briefly, frozen-sections may be prepared by (A) rehydrating 50 ng of frozen "pulverized" breast tumor at room temperature in PBS in small plastic capsules, (B) pelleting the particles by centrifugation, (C) resuspending them in a viscous embedding medium (OCT), (D) inverting the capsule and pelleting again by centrifugation, (E) snap-freezing in -70°C isopentane, (F) cutting the plastic capsule and removing the frozen

-72-

cylinder of tissue, (G) securing the tissue cylinder on a cryostat microtome chuck, and (H) cutting 25-50 serial sections containing an average of about 500 remarkably intact tumor cells.

- 5 Permanent-sections may be prepared by a similar method involving (A) rehydration of the 50 mg sample in a plastic microfuge tube, (B) pelleting, (C) resuspending in 10% formalin for 4 hours fixation, (D) washing/pelleting, (E) resuspending in warm 2.5% agar, (F) pelleting, (G) cooling in ice water to harden the agar, (H) removing the tissue/agar block
10 from the tube, (I) infiltrating and embedding the block in paraffin, and (F) cutting up to 50 serial permanent sections.

4. Immunoprecipitation

- 15 The antibodies of the present invention are particularly useful for the isolation of CD3 by immunoprecipitation. Immunoprecipitation involves the separation of the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Nonionic salts are preferred, since other agents such
20 as bile salts, precipitate at acid pH or in the presence of bivalent cations.

* * *

- 25 While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents
30 which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those

-73-

skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All claimed matter can be made without undue experimentation.

-74-

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-80-

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10 (E) COUNTRY: USA

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(ii) TITLE OF INVENTION: Methods and Materials For
Modulation of the Immuno-
suppressive Activity and
Toxicity of Monoclonal
Antibodies

15

(iii) NUMBER OF SEQUENCES: 17

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(v) COMPUTER READABLE FORM:

30

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PC-DOS/MS-DOS, ASCII

35

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Unknown

(B) FILING DATE: Concurrently herewith

(C) CLASSIFICATION: Unknown

40

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(A) APPLICATION NUMBER: 070.116

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(viii) ATTORNEY/AGENT INFORMATION:

45

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50

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-81-

(2) INFORMATION FOR SEQ ID NO:1:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 53..760
- 15 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1151..1186
- 20 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1308..1634
- 25 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1732..2055

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- 83 -

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-85-

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 1592 CAG GAC TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA
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-86-

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 40 45 50
 10 CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC CTC TAC AGC AGG CTA 1941
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu
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 Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser
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 20 GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACA CAG AAG AGC CTC TCC 2037
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-87-

CTCCCTCGGC AGGTTGGGGC ATTGGCCAGC GTGGCCCTCC CTCCAGCAGC AGGACTCTAG 2392
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5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 235 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 20
 Pro Gly Arg Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe 45
 35
 Thr Arg Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu 60
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 Glu Trp Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn 75
 65 80

30

Gln Lys Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
 85 90 95
 5 Thr Ala Phe Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val
 100 105 110
 Tyr Phe Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp
 115 120 125
 10 Gly Gln Gly Thr Pro Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
 130 135 140
 Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
 145 150 155 160
 Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
 165 170 175
 20 Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
 180 185 190
 Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
 195 200 205
 25 Val Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp
 210 215 220
 His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val
 225 230 235
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-89-

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 109 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
 1 5 10 15
 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 20 25 30

-90-

Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val
 35 40 45
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 5 50 55 60
 Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
 65 70 75 80
 Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
 85 90 95
 Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
 100 105
 (2) INFORMATION FOR SEQ ID NO:5:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 108 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
 Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu
 1 5 10 15
 Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
 20 25 30

-91-

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Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
 35      40
5      Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
      50      55      60
      Phe Leu Tyr Ser Arg Leu Thr Thr Val Asp Lys Ser Arg Trp Gln Glu Gly
      65      70      75      80
10     Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
      85      90      95
      Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
      100      105
15

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 107 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
 1      5      10      15
30

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[illegible]

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 108 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi)	SEQUENCE DESCRIPTION:	SEQ ID NO:7:
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-93-

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly 15
 5 10
 5 Asp Arg Val Thr Ile Thr Cys Gln Ala Ser Gln Asp Ile Ile Lys Tyr 30
 20 25
 Leu Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Ile 45
 35 40
 10 Tyr Glu Ala Ser Asn Leu Gln Ala Gly Val Pro Ser Arg Phe Ser Gly 60
 50 55
 Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro 80
 65 70 75
 15 Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Gln Ser Leu Pro Tyr 95
 85 90
 Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg 105
 100

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-94-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

1  Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
5  Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
10 Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
15 Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
20 Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg

```

25 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 107 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-95-

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

5  Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Ser Leu Ser Ala Ser Val Gly
   1      5      10      15
10 Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Ser Tyr Met
   20      25      30      35      40      45
15 Asn Trp Tyr Gln Gln Thr Pro Gly Lys Ala Pro Lys Arg Trp Ile Tyr
   35      40      45      50      55      60      65
20 Asp Thr Ser Lys Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
   50      55      60      65      70      75      80
   Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
   65      70      75      80      85      90      95
25 Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser Asn Pro Phe Thr
   85      90      95      100      105
   Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
   100      105

```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 119 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

5 Gln Val Gln Leu Gln Gln Ser Gly Ala Gln Leu Ala Arg Pro Gly Ala
 1 5 10 15
 10 Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
 20 25 30
 15 Thr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
 50 55 60
 20 Lys Asp Lys Ala Thr Leu Thr Thr Asp Lys Ser Ser Ser Thr Ala Tyr
 65 70 75 80
 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
 85 90 95
 25 Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Thr Leu Thr Val Ser Ser
 115

30

-97-

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 126 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

15 Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Phe Ile Phe Ser Ser Tyr
 20 20 25 30
 Ala Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ala Ile Ile Trp Asp Asp Gly Ser Asp Gln His Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
 65 70 75 80
 Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
 85 90 95

-98-

Ala Arg Asp Gly Gly His Gly Phe Cys Ser Ser Ala Ser Cys Phe Gly
 100 105 110
 5 Pro Asp Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ser
 115 120 125

(2) INFORMATION FOR SEQ ID NO:12:

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20 Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15
 25 Ser Leu Arg Leu Ser Cys Ser Ser Ser Gly Tyr Thr Phe Thr Arg Tyr
 20 25 30
 Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 30 Ala Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
 50 55 60

-99-

5 Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
 65 70 75 80
 Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe Cys
 85 90 95
 Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
 100 105 110
 10 Thr Pro Val Thr Val Ser Ser
 115
 (2) INFORMATION FOR SEQ ID NO:13:
 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 119 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
 25 Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
 20 25 30
 30 Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45

-100-

5 Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
 50 55 60
 Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
 65 70 75 80
 Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 10 Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
 100 105 110
 Thr Pro Val Thr Val Ser Ser
 115
 15
 (2) INFORMATION FOR SEQ ID NO:14:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 119 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20
 (ii) MOLECULE TYPE: protein
 25
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
 30 Gln Val Gln Leu Val Gln Ser Gly Gly Val Val Gln Pro Gly Arg
 1 5 10 15

-101-

Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr
 20 25 30
 5 Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
 35 40 45
 Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys Phe
 50 55 60
 10 Lys Asp Arg Phe Thr Ile Ser Thr Asp Lys Ser Lys Ser Thr Ala Phe
 65 70 75 80
 Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln Gly
 100 105 110
 20 Thr Pro Val Thr Val Ser Ser
 115

(2) INFORMATION FOR SEQ ID NO:15:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

-102-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCAGATGTT AACTGCTCAC

20

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGGGGCCAG TGGATGGATA GAC

23

20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCCGCCACC

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-104-

CLAIMS:

1. A monoclonal antibody, the antibody further defined as "humanized" murine OKT3 antibody having a human Fc region and a murine antigen binding region, wherein the antibody has a mutated Fc receptor binding region and reduced T cell activating properties relative to murine OKT3.
2. The monoclonal antibody of claim 1, wherein the antibody comprises a mutation at position 235 of the "humanized" murine OKT3 antibody.
3. The monoclonal antibody of claim 2, comprising a mutation from a leucine to a glutamic acid at position 235.
4. The monoclonal antibody of claim 2, comprising a mutation from a leucine to an alanine at position 235.
5. The monoclonal antibody of claim 1, comprising a mutation at position 234.
6. The monoclonal antibody of claim 5, comprising a mutation from a phenylalanine to an alanine at position 234.

-105-

7. The monoclonal antibody of claim 5, comprising a mutation from a phenylalanine to an alanine at position 234 and a second mutation from a leucine to an alanine at position 235.

5

8. The monoclonal antibody of claim 1, wherein the human Fc region is an IgG1 or an IgG4 Fc portion.

10

9. The monoclonal antibody of claim 8, wherein the human Fc region is an IgG1.

15

10. The monoclonal antibody of claim 8, wherein the antibody comprises a mutation at position 235 of the "humanized" murine OKT3 antibody.

20

11. The monoclonal antibody of claim 10, wherein the mutation is from a leucine to a glutamic acid at position 235.

25

12. The monoclonal antibody of claim 10, comprising a mutation from a leucine to an alanine at position 235.

30

13. The monoclonal antibody of claim 8, comprising a mutation at position 234.

-106-

14. The monoclonal antibody of claim 13, comprising a mutation from a phenylalanine to an alanine at position 234.

5 15. The monoclonal antibody of claim 13, comprising a mutation from a phenylalanine to an alanine at position 234 and a second mutation from a leucine to an alanine at position 235.

10 16. A pharmaceutical composition comprising the monoclonal antibody of claim 1 and a physiologically acceptable carrier.

15 17. A method of suppressing immune response-triggered rejection of transplanted organ tissue comprising the step of administering to an organ transplant patient, either before, during or after transplantation, a monoclonal antibody of claim 1 in a physiologically acceptable carrier.

20 18. A method for suppression of immune response-triggered rejection of transplanted organ tissue comprising the step of administering to an organ transplant patient, either before, during or after transplantation, a pharmaceutical composition that comprises an antibody
25 according to claim 1, and a physiologically acceptable carrier, wherein the antibody modulates immune response through binding to a first T-cell surface protein, designated CD3, and, simultaneously, to a second T-cell surface protein.

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-107-

19. The method of claim 18, wherein the second T-cell surface protein is selected from the group consisting of CD3, CD4, and CD8.

1/24

	1	10	20	30	40	50	60
Ok3vl	QIVLTQSPAIMSASPGEKVTITCSASS-SYDMMWYQOKSGTSPKRWIYDTSKLASGVPA						
REI	DIQMTQSPSSLSASVGDRTITTCQASGRIILWYQOTPGKAPKILTYEASNLOAGVPS						
gISA-S-SVS-M.....ET-X-AS.....						
gICSA-S-SVS-M.....RM-ET-X-AS.....						
	70	80	90	100	108		
Ok3vl	HFRGSGSGTYSYSLTISGHEADAATYYQOMSENPEYFGSOTKLEINR						
REI	HFGSGSGTDYTFPTISSLOPEDLATYYCQOYCSLEKTFGGTKLQITR						
gIANS-N-F.....						
gICNS-N-F.....						

FIGURE 1A

	1	10	20	30	40	50	a	60
Ok3vh	QVGLQSGAKLARPCASVTHSCIKASCTTITKTMHWKQRPCCGLEWTCYTHPSGCTTRDN							
KOL	QVQLVESGGGVVQPGRELRLSCSSSGTIFPSYAMHWVRQAPGKGLDVALEINDDGSDQHYA							
gHY-T-T-T-N.....Y-NPSKQCTN-N							
gHAQ.....Y-T-T-T-N.....IGY-NPSKQCTN-N							
gHGQ.....Y-T-T-T-N.....IGY-NPSKQCTN-N							
	70	80	abc	90	100	abcdefghi	110	
Ok3vh	QKPKDKATLTDRSSSTAYMQLSSELTSEDSAVYYCARQYGDHYCL-----DQWQCTTLTVES							
KOL	DSYKGRFTISRDNKQNTLFQMDSLRPEDTCVYPCARFGGCHGRCSSASCTGEMYNQOCTPVTVES							
gH	QK-P.....YGDHY-L-----							
gHA	QK-P.....YGDHY-L-----							
gHG	QK-P.....YGDHY-L-----							

FIGURE 1B

2/24

5 10 15 20 25 30 35 40 45 50 55
 ATCCTGGCAA AGATTGTAAT ACGACTCACT ATAGGGCGAA TTCGCCGCCA CC ATG GAA
 Met Glu>
 ____a____>

60 65 70 75 80 85 90 95 100 105
 TGG AGC TGG GTC TTT CTC TTC TTC CTG TCA GTA ACT ACA GGT GTC CAC
 Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly Val His>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

110 115 120 125 130 135 140 145 150
 TCC CAG GTT CAG CTG GTG CAG TCT GGA GGA GGA GTC GTC CAG CCT GGA
 Ser Gln Val Gln Leu Val Gln Ser Gly Gly Gly Val Val Gln Pro Gly>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

155 160 165 170 175 180 185 190 195 200
 AGG TCC CTG AGA CTG TCT TGT AAG GCT TCT GGA TAC ACC TTC ACT AGA
 Arg Ser Leu Arg Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

205 210 215 220 225 230 235 240 245 250
 TAC ACA ATG CAC TGG GTC AGA CAG GCT CCT GGA AAG GGA CTC GAG TGG
 Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

255 260 265 270 275 280 285 290 295
 ATT GGA TAC ATT AAT CCT AGC AGA GGT TAT ACT AAC TAC AAT CAG AAG
 Ile Gly Tyr Ile Asn Pro Ser Arg Gly Tyr Thr Asn Tyr Asn Gln Lys>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

300 305 310 315 320 325 330 335 340 345
 GTG AAG GAC AGA TTC ACA ATT TCT AGA GAC AAT TCT AAG AAT ACA GCC
 Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Ala>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

350 355 360 365 370 375 380 385 390
 TTC CTG CAG ATG GAC TCA CTC AGA CCT GAG GAT ACC GGA GTC TAT TTT
 Phe Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Gly Val Tyr Phe>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

395 400 405 410 415 420 425 430 435 440
 TGT GCT AGA TAT TAC GAT GAC CAC TAC TGT CTG GAC TAC TGG GGC CAA
 Cys Ala Arg Tyr Tyr Asp Asp His Tyr Cys Leu Asp Tyr Trp Gly Gln>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)____a____a____>

>SEQED_(include)_of:_ja91.ins_check:_5694_from:_1_to:_3153

445 450 455 460 465 470 475 480 485 490
 GGT ACC CCG GTC ACC GTG AGC TCA GGT TCC ACC AAG GGC CCA TCC CTC
 Gly Thr Pro Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val>

FIGURE 2A

3/24

____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____
 495 500 505 510 515 520 525 530 535
 TTC CCC CTG GCG CCC TGC TCC AGG AGC ACC TCC GAG AGC ACA GCC GCC
 Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____>
 540 545 550 555 560 565 570 575 580 585
 CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG
 Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____>
 590 595 600 605 610 615 620 625 630
 TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC
 Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____>
 635 640 645 650 655 660 665 670 675 680
 CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC
 Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____>
 685 690 695 700 705 710 715 720 725 730
 TCC AGC AGC TTG GGC ACG AAG ACC TAC ACC TGC AAC GTA GAT CAC AAG
 Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His Lys>
 ____a____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]____a____a____>
 735 740 745 750 755 760 765 770 775 780
 CCC AGC AAC ACC AAG GTG GAC AAG AGA GTT GGTGAGAGGC CAGCACAGGG
 Pro Ser Asn Thr Lys Val Asp Lys Arg Val>
 ____TRANSLATION OF OKT3 HC IGG4 12/4____>
 785 790 795 800 805 810 815 820 825 830 835 840
 AGGGAGGGTG TCTGCTGGAA GCCAGGCTCA GCCCTCCTGC CTGGACGCAC CCCGGCTGTG
 845 850 855 860 865 870 875 880 885 890 895 900
 CAGCCCCAGC CCAGGGCAGC AAGGCATGCC CCATCTGTCT CCTCACCCGG AGGCCTCTGA
 905 910 915 920 925 930 935 940 945 950 955 960
 CCACCCCACT CATGCTCAGG GAGAGGGTCT TCTGGATTTT TCCACCAGGC TCCCGGCACC
 965 970 975 980 985 990 995 1000 1005 1010 1015 1020
 ACAGGCTGGA TGCCCTTACC CCAGGCCCTG CGCATAACAGG GCAGGTGCTG CGCTCAGACC
 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080
 TGCCAAGAGC CATATCCGGG AGGACCCTGC CCCTGACCTA AGCCACCCCC AAAGGCCAAA
 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140
 CTCTCCACTC CCTCAGCTCA GACACCTTCT CTCCTCCAG ATCTGAGTAA CTCCCAATCT

FIGURE 2B

4/24

1145 1150 1155 1160 1165 1170 1175 1180 1185 1190
 TCTCTCTGCA GAG TCC AAA TAT GGT CCC CCA TGC CCA TCA TGC CCA GGTA
 Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro>
 ____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_b____>

1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250
 AGCCCAACCCA GGCCTCGCCC TCCAGCTCAA GCGGGGACAG GTGCCCTAGA GTAGCCTGCA
 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310
 TCCAGGGACA GGCCCCAGCC GGGTGGTGAC GCATCCACCT CCATCTCTTC CTCAGCA CCT
 Pro>
 ____>

1315 1320 1325 1330 1335 1340 1345 1350 1355
 GAG TTC CTG GGG GGA CCA TCA GTC TTC CTG TTC CCC CCA AAA CCC AAG
 Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1360 1365 1370 1375 1380 1385 1390 1395 1400 1405
 GAC ACT CTC ATG ATC TCC CGG ACC CCT GAG GTC ACG TGC GTG GTG GTG
 Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1410 1415 1420 1425 1430 1435 1440 1445 1450
 GAC GTG AGC CAG GAA GAC CCC GAG GTC CAG TTC AAC TGG TAC GTG GAT
 Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1455 1460 1465 1470 1475 1480 1485 1490 1495 1500
 GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTC
 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1505 1510 1515 1520 1525 1530 1535 1540 1545 1550
 AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC
 Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val His Gln Asp>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1555 1560 1565 1570 1575 1580 1585 1590 1595
 TGG CTG AAC GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GGC CTC
 Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu>
 ____c____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____c____>

1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650
 CCG TCC TCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGTGGG ACCCAGGGG
 Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys>
 ____TRANSLATION OF OKT3 HC IGG4 12/4/92 (A)_c____>

1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710
 TGCGAGGGCC ACACGGACAG AGGCCAGCTC GGCCCAACCT CTGCCCTGGG AGTGACCCCT

FIGURE 2C

5/24

1715 1720 1725 1730 1735 1740 1745 1750 1755 1760
 GTGCCAACCT CTGTCCCTAC A GGG CAG CCC CGA GAG CCA CAG GTG TAC ACC
 Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr>
 _____TRANSLATION OF OKT3 HC IGG4 12/4____>

1765 1770 1775 1780 1785 1790 1795 1800 1805
 CTG CCC CCA TCC CAG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC
 Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

1810 1815 1820 1825 1830 1835 1840 1845 1850 1855
 TGC CTG GTC AAA GGC TTC TAC CCC AGC GAC ATC GCC GTG GAG TGG GAG
 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

1860 1865 1870 1875 1880 1885 1890 1895 1900 1905
 AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG
 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

1910 1915 1920 1925 1930 1935 1940 1945 1950
 GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AGG CTA ACC GTG GAC AAG
 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

1955 1960 1965 1970 1975 1980 1985 1990 1995 2000
 AGC AGG TGG CAG GAG GGG AAT GTC TTC TCA TGC TCC GTG ATG CAT GAG
 Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

2005 2010 2015 2020 2025 2030 2035 2040 2045
 GCT CTG CAC AAC CAC TAC ACA CAG AAG AGC CTC TCC CTG TCT CTG GGT
 Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly>
 _____d_____TRANSLATION OF OKT3 HC IGG4 12/4/92 [A]_____d_____>

2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100
 AAA TGA GTGCC AGGGCCGGCA AGCCCCCGCT CCCCAGGGCTC TCGGGGTCGC
 Lys ***>
 _____d_____>

2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160
 GCGAGGATGC TTGGCACGTA CCCCCTCTAC ATACTTCCCA GGCACCCAGC ATGGAATAA

2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220
 AGCACCCACC ACTGCCCTGG GCCCCTGTGA GACTGTGATG GTTCTTTCCA CGGGTCAGGC

2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280
 CGAGTCTGAG GCCTGAGTGA CATGAGGGAG GCAGAGCGGG TCCCACTGTC CCCACACTGG

2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340

FIGURE 2D

6/24

CCCAGGCCTT GCAGTGTGTC CTGGGCCACC TAGGGTGGG CTCAGCCAGG GGCTCCCTCG
2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395
GCAGGGTGGG GCATTTGCCA GCGTGGCCCT CCGTCCAGCA GCAGGACTCT AGAGGATCC

FIGURE 2E

7/24

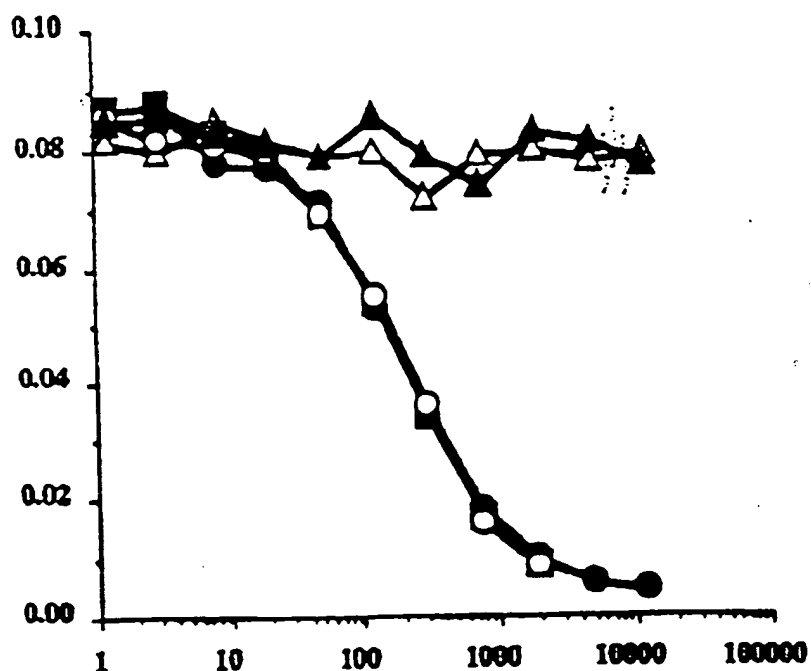


FIGURE 3A

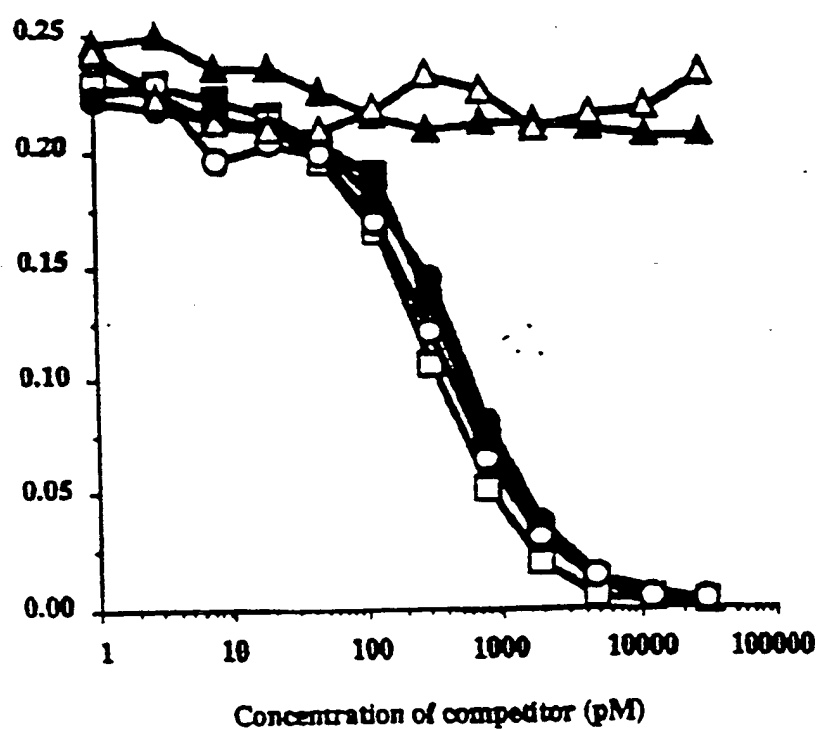


FIGURE 3B

8/24

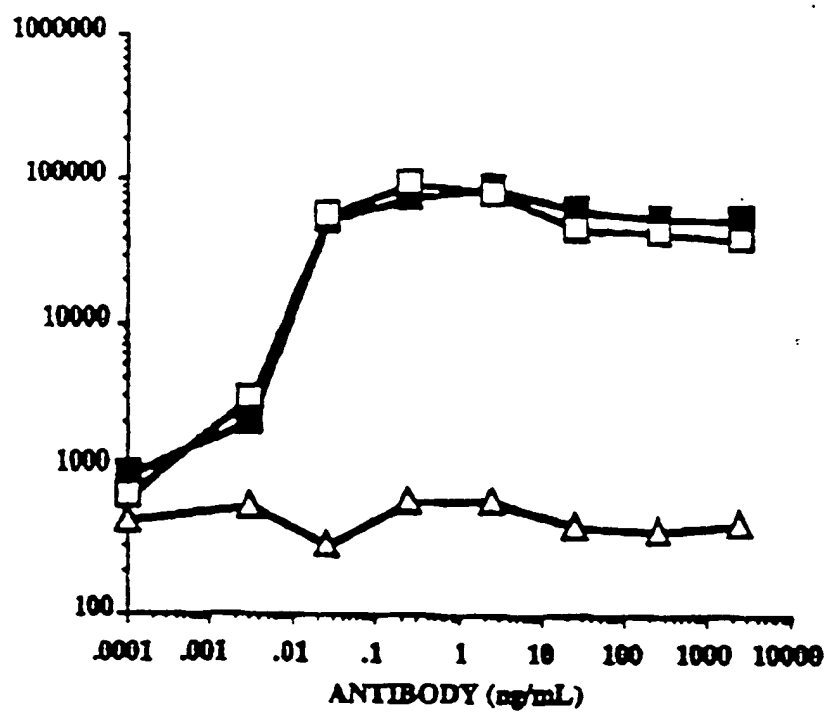


FIGURE 4

9/24

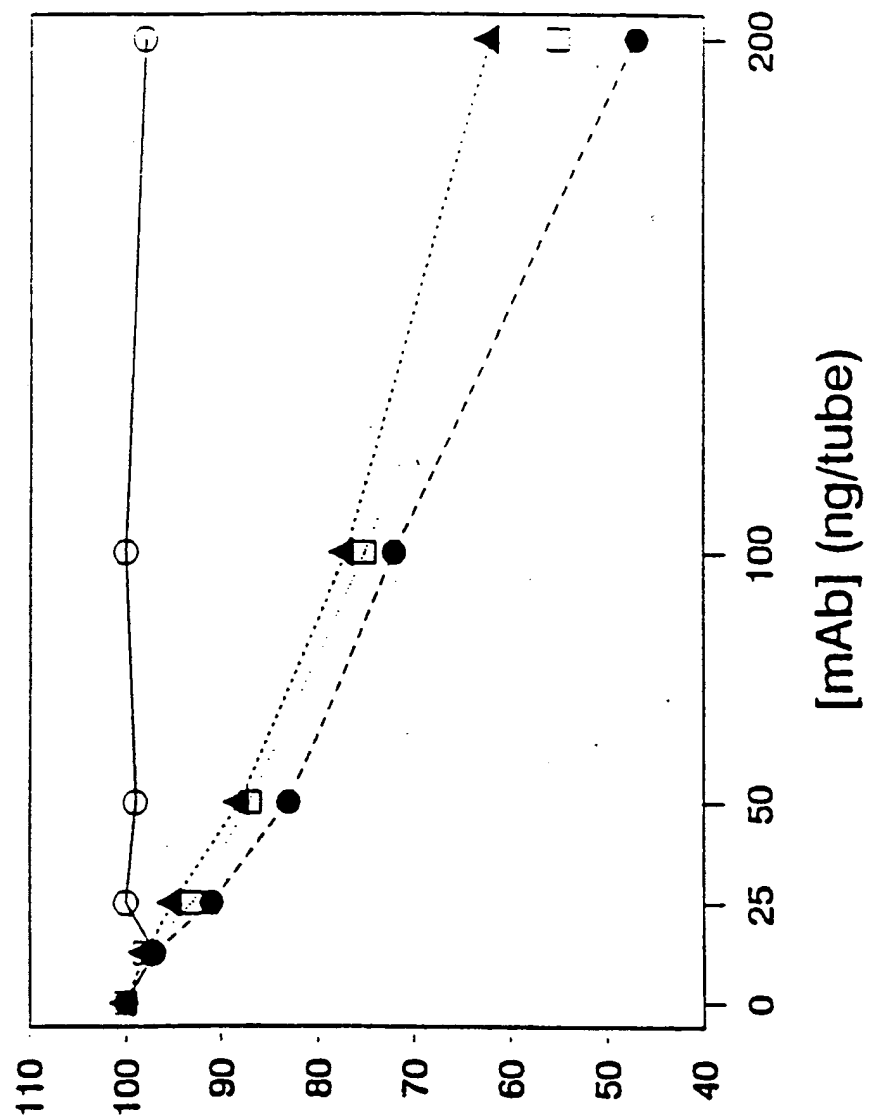


FIGURE 5

10/24

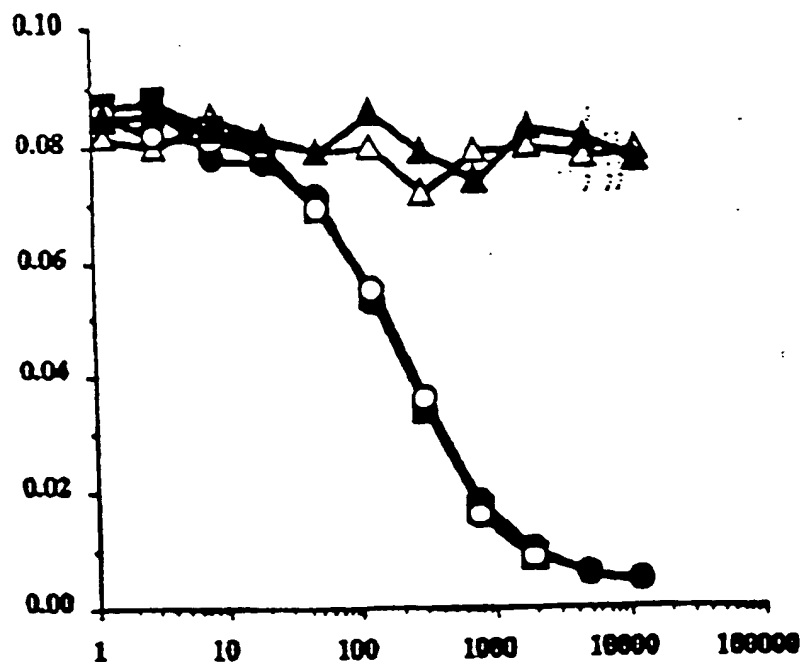
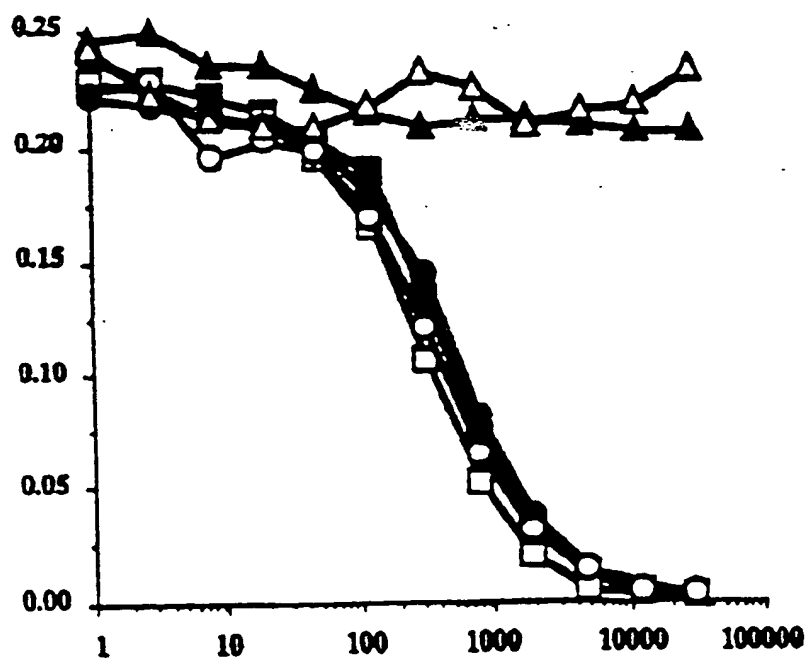


FIGURE 6A



Concentration of competitor (pM)

FIGURE 6B

11/24

Isotype	234	235	236	237	238	239
hlgG1 hlgG3 mlgG2a	<u>Leu</u>	<u>Leu</u>	Gly	Gly	Pro	Ser
hlgG4	<u>Phe</u>	<u>Leu</u>	Gly	Gly	Pro	Ser
hlgG2	<u>Val</u>	---	Ala	Gly	Pro	Ser
mlgG2b	<u>Leu</u>	<u>Glu</u>	Gly	Gly	Pro	Ser
hlgG1A/A	<u>Ala</u>	<u>Ala</u>	Gly	Gly	Pro	Ser
hlgG4A/A	<u>Ala</u>	<u>Ala</u>	Gly	Gly	Pro	Ser

FIGURE 7

12/24

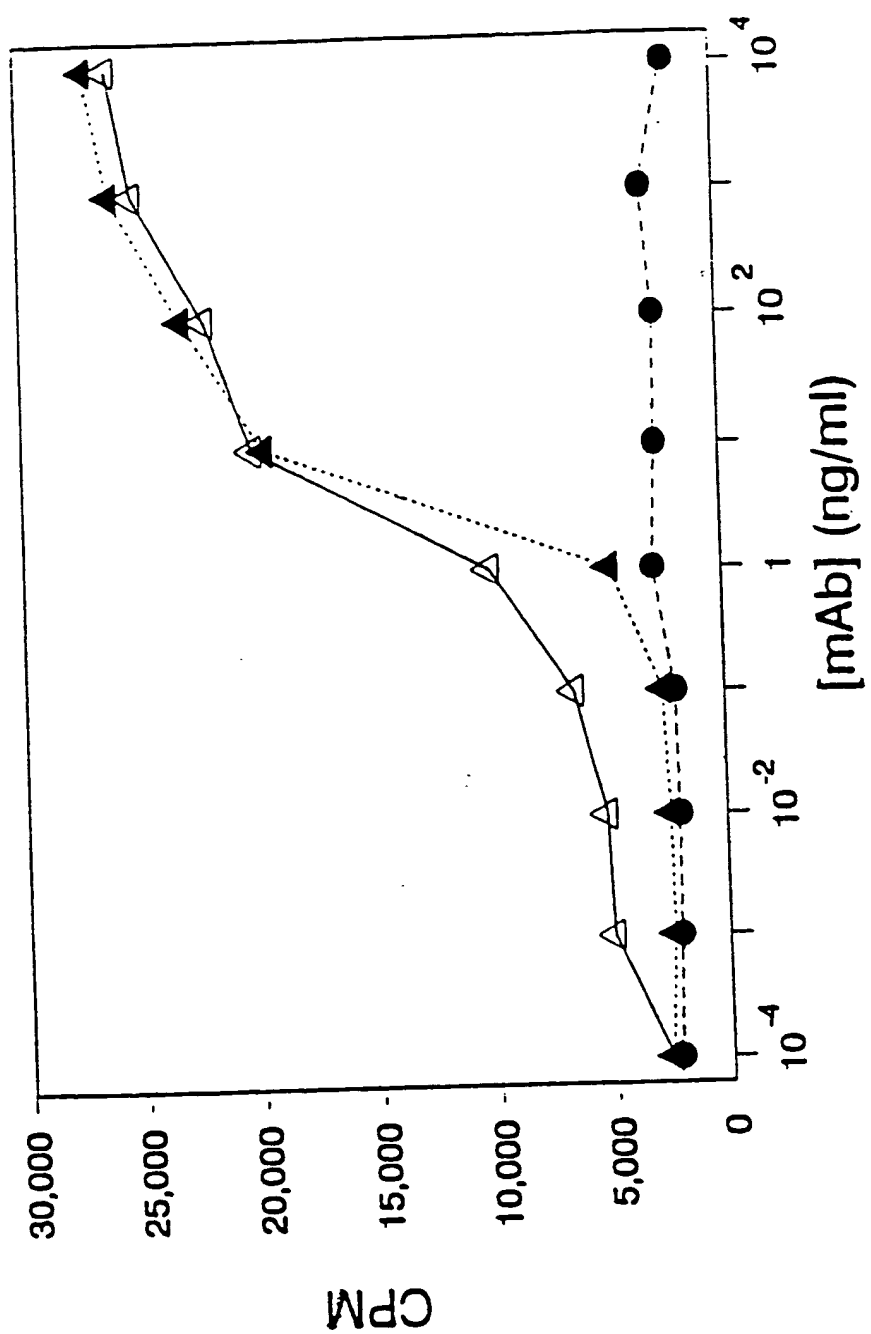


FIGURE 8

13/24

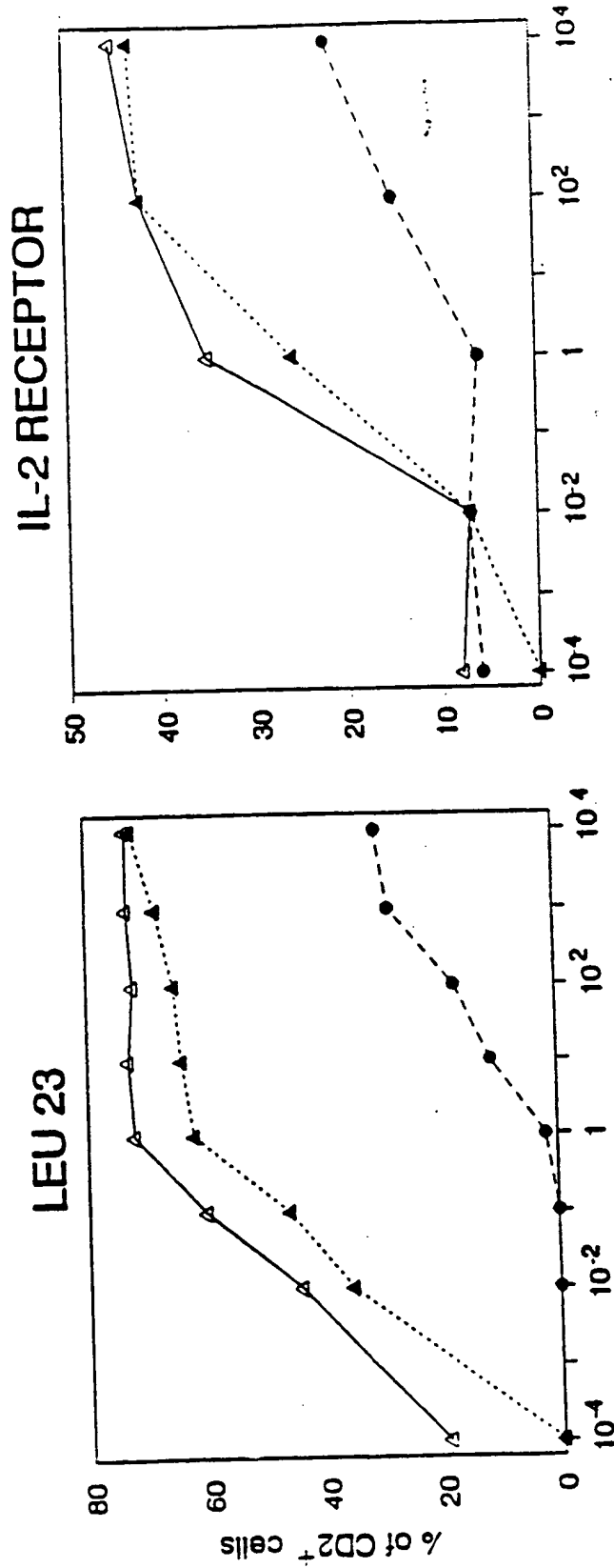


FIGURE 9A

[mAb] (ng/ml)

FIGURE 9B

14/24

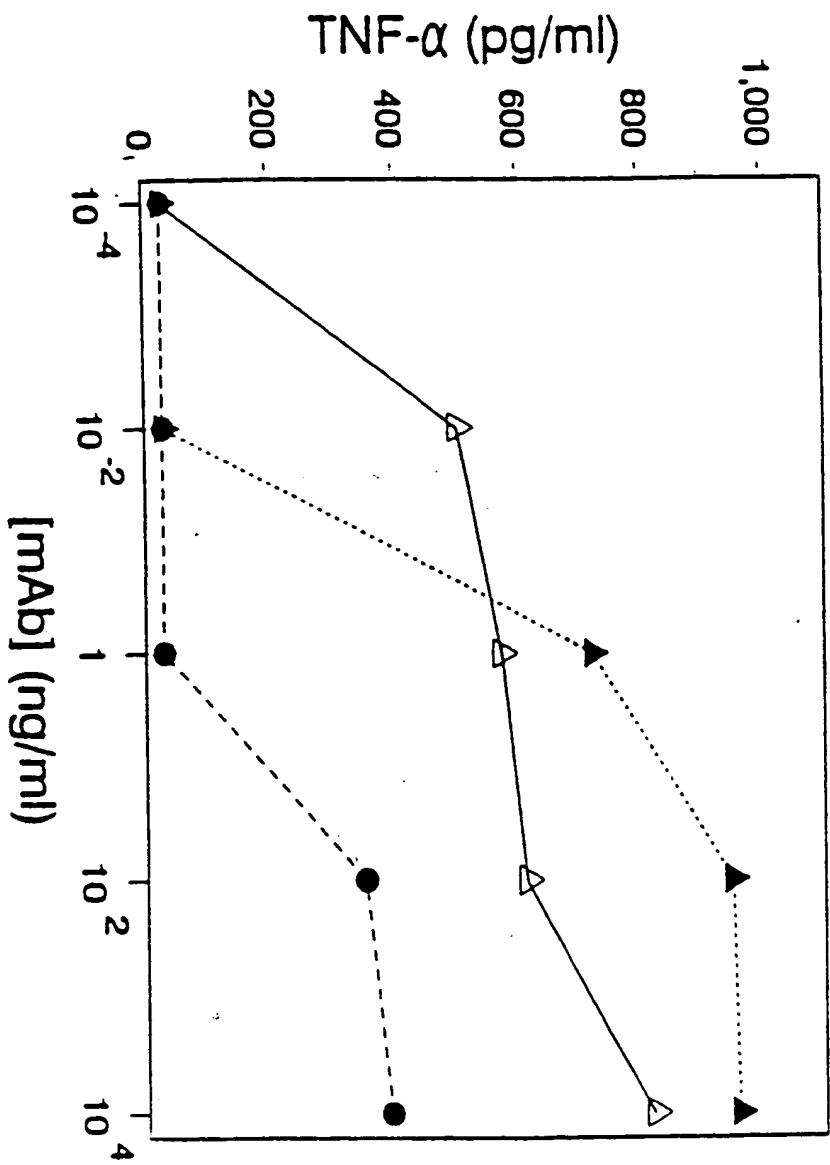


FIGURE 10

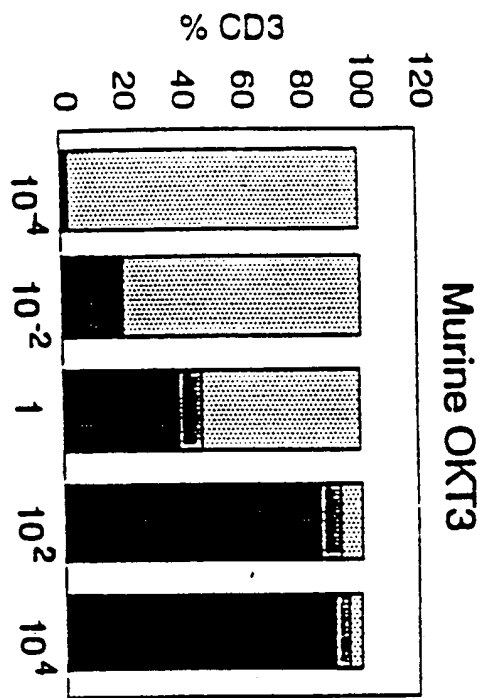
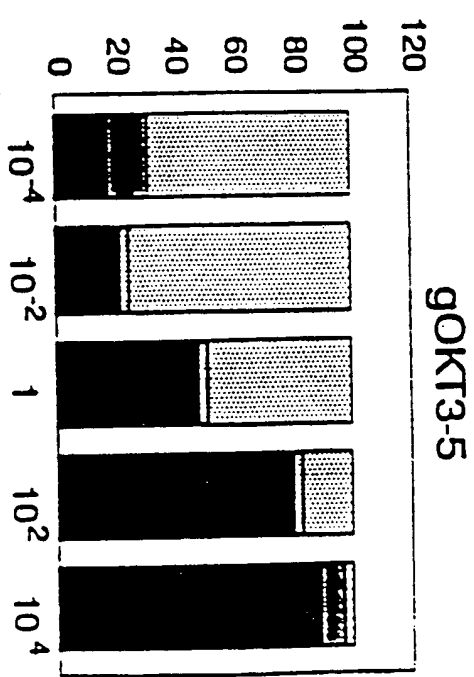


FIGURE 11A



Glu-235

FIGURE 11B

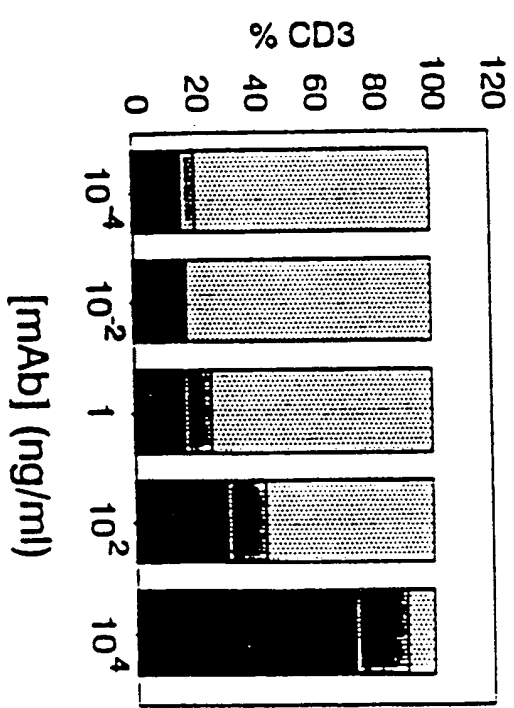


FIGURE 11C

[mAb] (ng/ml)

16/24

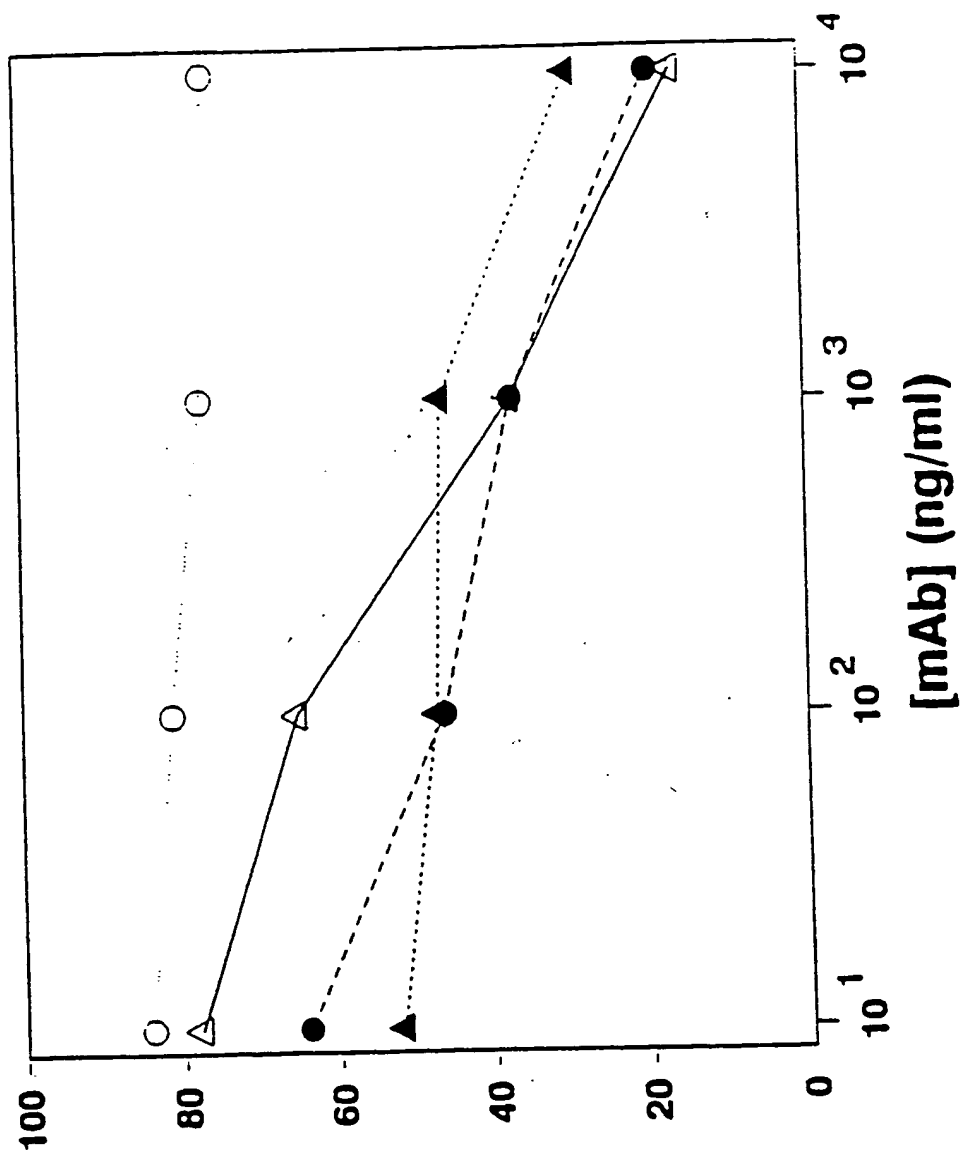


FIGURE 12

17/24

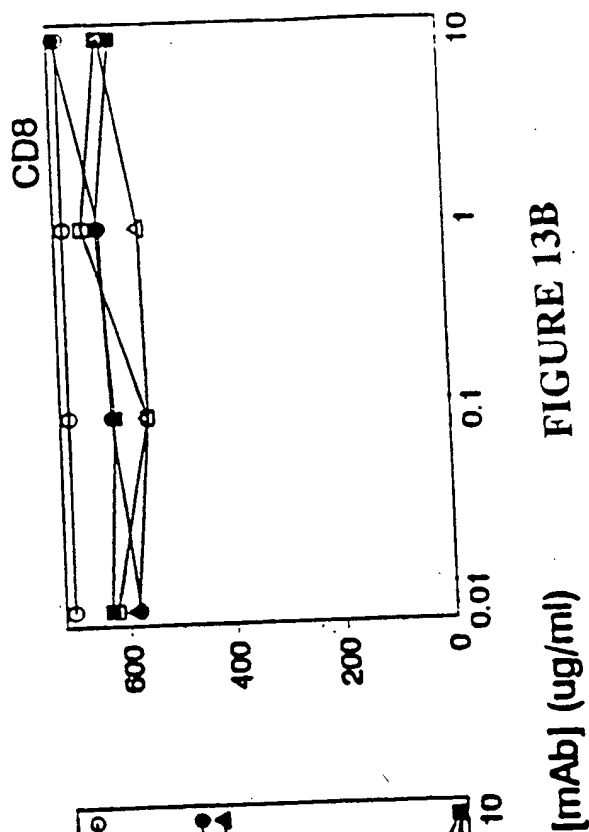


FIGURE 13A

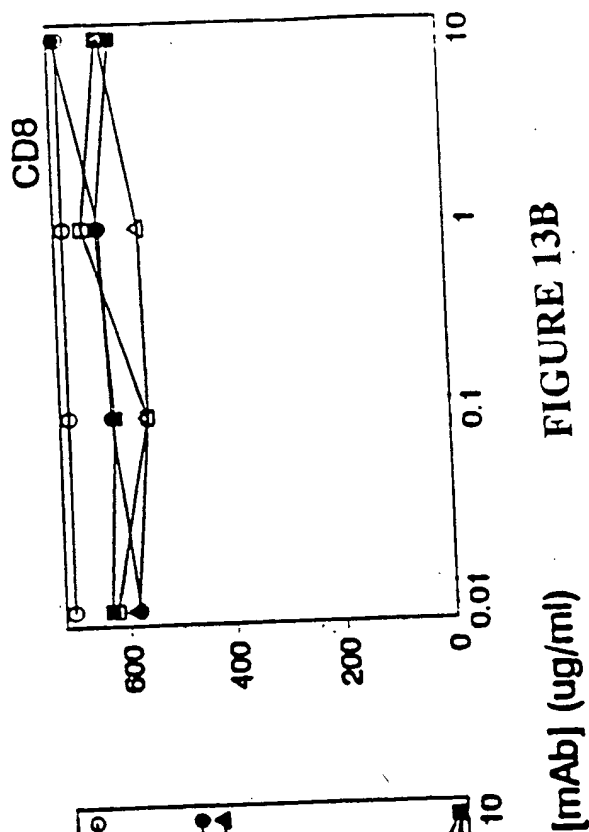


FIGURE 13B

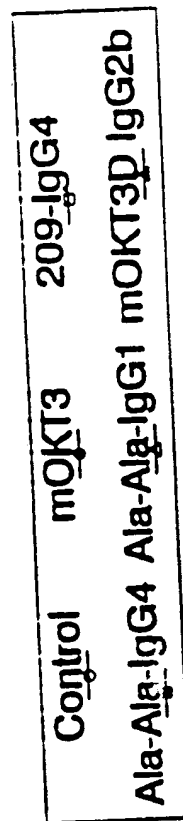


FIGURE 13C

18/24

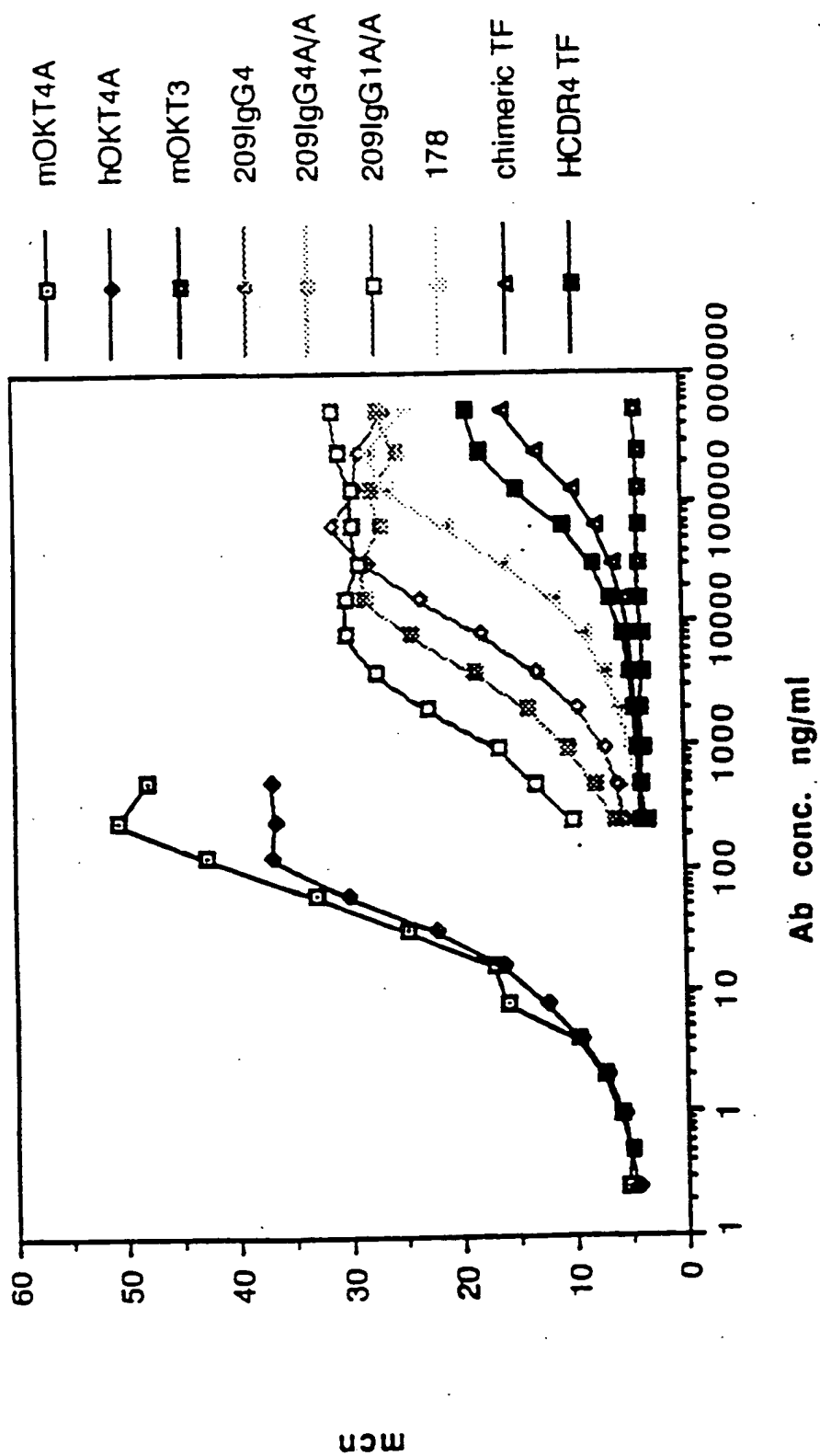


FIGURE 14

19/24

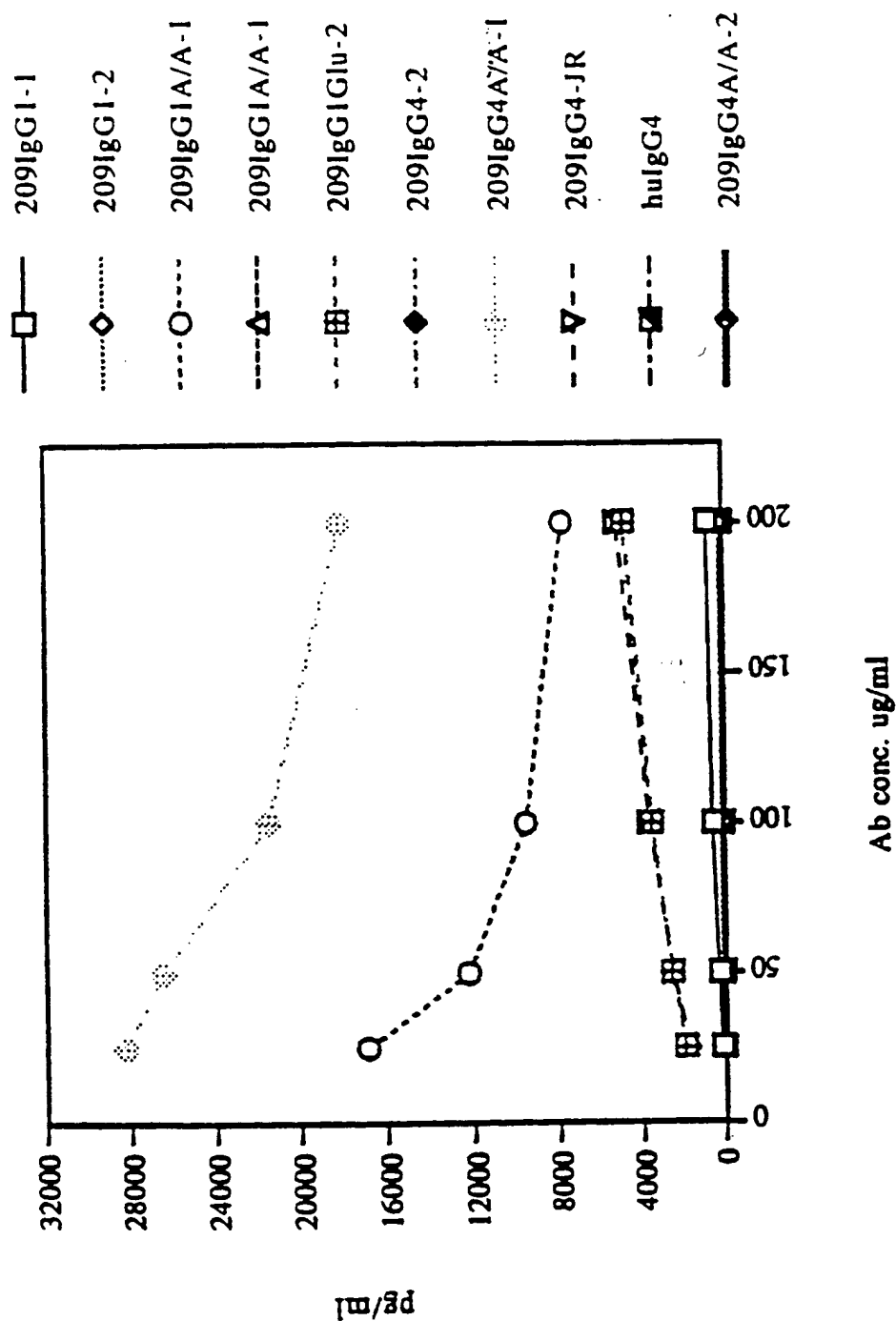


FIGURE 15

20/24

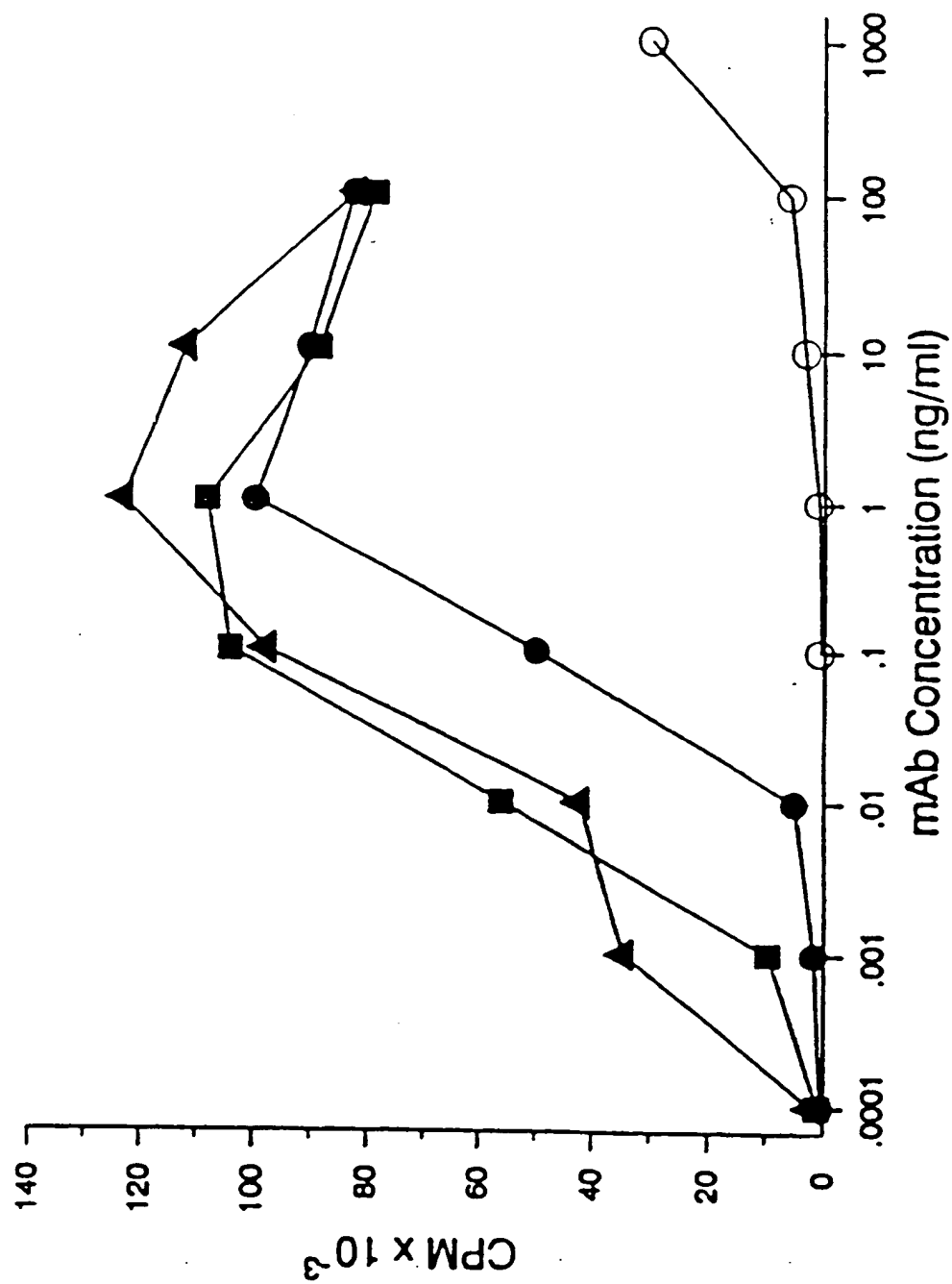
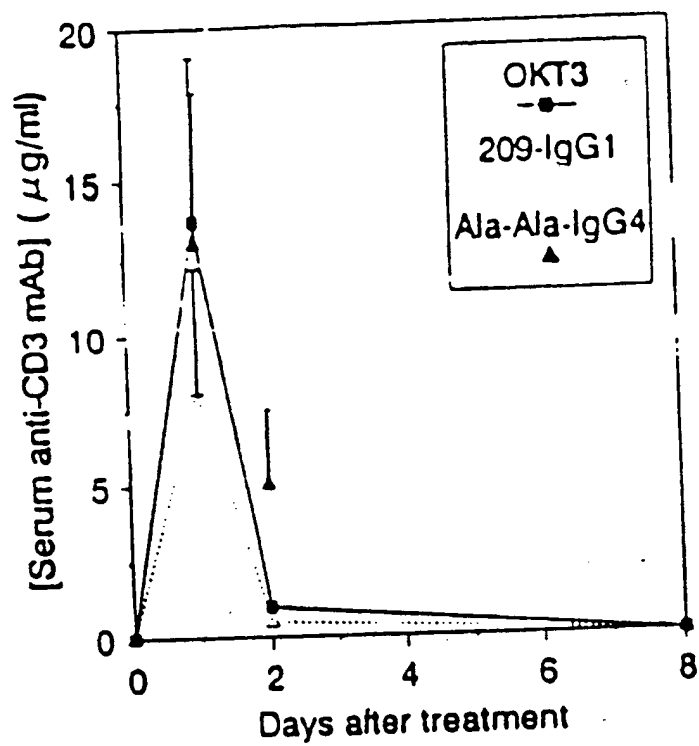
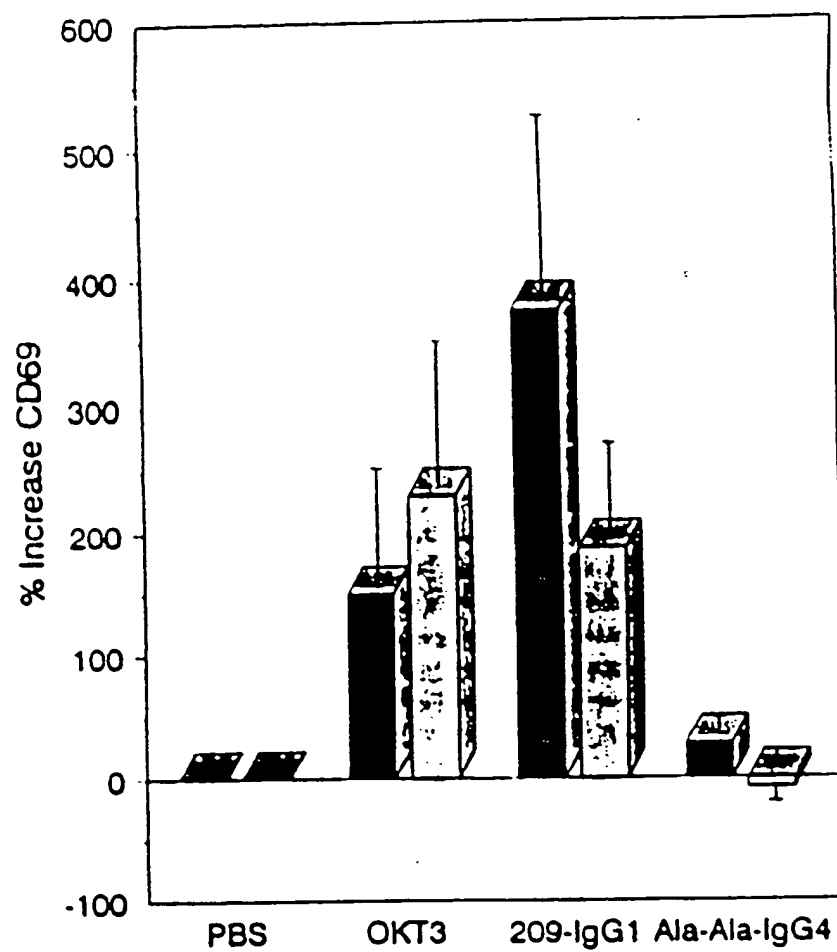


FIGURE 16

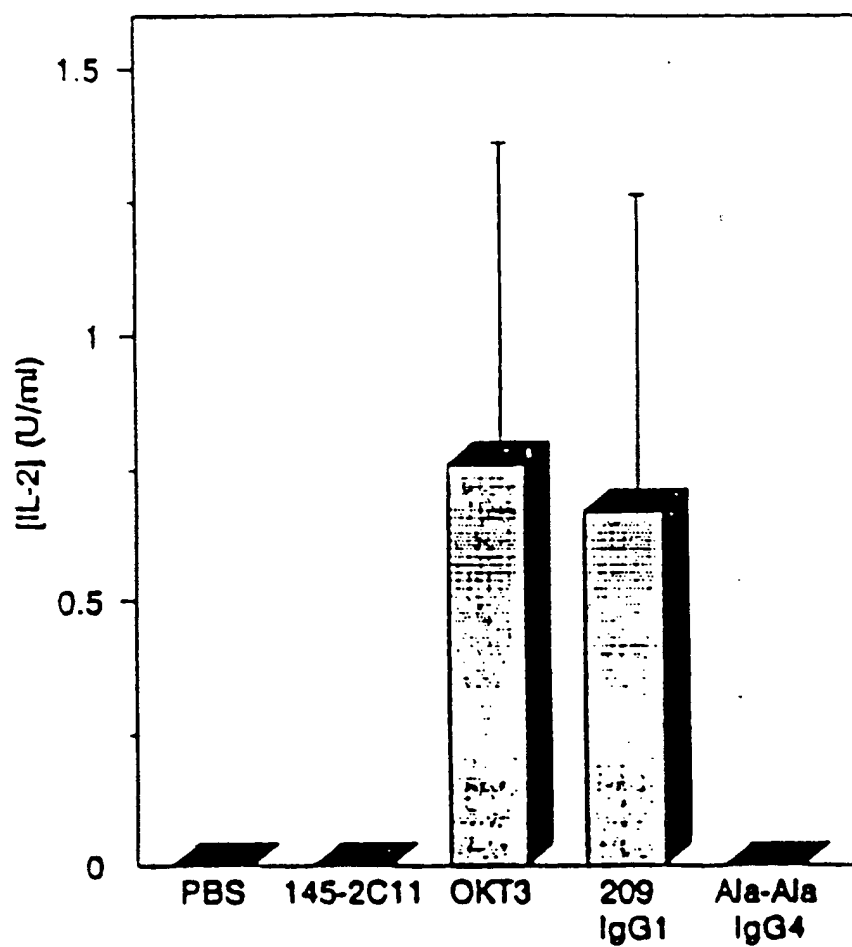
21/24

**FIGURE 17**

22/24

**FIGURE 18**

23/24

**FIGURE 19**

24/24

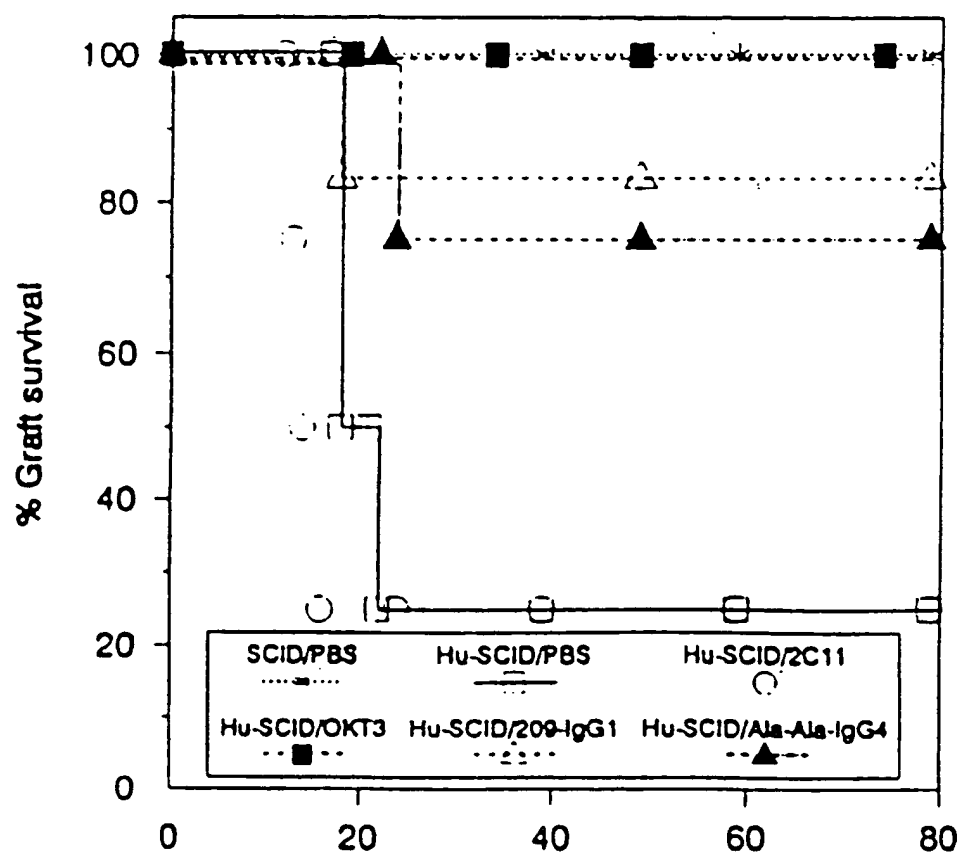


FIGURE 20

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/06198

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 5 C07K15/28 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF IMMUNOLOGY, vol.148, no.11, 1 June 1992, BALTIMORE MD, USA pages 3461 - 3468 M. ALEGRE ET AL. 'Effect of a single amino acid mutation on the activating and immunosuppressive properties of a ' cited in the application see the whole document</p> <p style="text-align: center;">--- -/--</p>	1-17

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

A document member of the same patent family

Date of the actual completion of the international search

20 October 1994

Date of mailing of the international search report

02 -11- 1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Appli n No

PCT/US 94/06198

Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Citation of document, with indication, where appropriate, of the relevant passages		Relevant to claim No.
X	<p>THE JOURNAL OF IMMUNOLOGY, vol.148, no.9, 1 May 1992, BALTIMORE MD, USA pages 2756 - 2763 E. WOODLE ET AL. 'Humanized OKT3 antibodies: Successful transfer of immune modulating properties and idiotype expression.' cited in the application see abstract see page 2761, right column, line 50 - page 2762, left column, line 13 ---</p>	1-17
X	<p>EUROPEAN JOURNAL OF IMMUNOLOGY, vol.23, no.2, February 1993, WEINHEIM, GERMANY pages 403 - 411 S. BOLT ET AL. 'The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties.' see abstract ---</p>	1-17
X	<p>INTERNATIONAL REVIEW OF IMMUNOLOGY, vol.10, no.2-3, 1993 pages 241 - 250 L. JOLLIFFE 'Humanized antibodies: Enhancing therapeutic utility through antibody engineering.' see the whole document ---</p>	1-17
A	<p>WO,A,91 09968 (CELLTECH LIMITED) 11 July 1991 see page 13, line 34 - page 14, line 11 see claims -----</p>	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/06198

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 17-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/06198

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9109968	11-07-91	AU-B- 646009	03-02-94
		AU-A- 6974091	24-07-91
		AU-B- 649645	02-06-94
		AU-A- 7033091	24-07-91
		AU-B- 631481	26-11-92
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		EP-A- 0460178	11-12-91
		EP-A- 0620276	19-10-94
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		WO-A- 9109967	11-07-91
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		GB-A, B 2268744	19-01-94
		GB-A, B 2268745	19-01-94
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		JP-T- 5500312	28-01-93
